INVESTIGATION OF COMPLEMENT PROTEIN C1Q – IMPLICATIONS FOR ITS PROTECTIVE ROLES AGAINST SYSTEMIC LUPUS ERYTHEMATOSUS

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Summary

C1q is an abundant plasma protein and is the first component of the complement classical pathway. It binds to antibody-opsonized microbial pathogens or certain pathogenic self antigens and initiates the activation of the complement classical pathway. It is also known to have diverse functions beyond providing immunity against pathogens, and is implicated in the pathogenesis of diseases such as transmissible spongiform encephalopathy, Alzheimer's disease and familial dementia. Conversely, hereditary C1q deficiency in human almost always leads to the autoimmune condition known as systemic lupus erythematosus (SLE), and lupus-like conditions also developed in $C1q^{-/-}$ mice. In addition, SLE itself causes consumption of C1q in patients who can produce C1q normally, and these patients also developed anti-C1q antibodies that can deplete bioavailable C1q.

C1q is produced by dendritic cells (DCs) and macrophages, the two main types of antigen presentation cells, and DCs are particularly important in the maintenance of tolerance as well as induction of immunity. In view of the strong association of C1q and DCs with autoimmune SLE conditions, we investigated the regulation of C1q production in DCs. We have developed assays to quantitate cellular C1q mRNA, protein expression and also developed an ELISA assay for measuring secreted C1q in the DC culture. By ELISA, we screened a large number of stimuli for their ability to modulate C1q production in DCs. Marked downregulation of C1q production was observed by two stimuli, i.e. zymosan and interferon alpha (IFN-α). On the other hand, IFN-γ was found to be a potent inducer of C1q production.

In terms of the signaling mechanisms involved, we found that zymosan signals through the Dectin-1 receptor to mediate the downregulation of C1q production. It resulted in a thorough reduction in C1q mRNA, cellular protein and secreted protein. In contrast, IFN-α upregulated C1q mRNA and cellular protein levels, but it reduced the secretion of C1q by DCs after prolonged treatments. In this case, we found that C1q was mainly trapped in the endoplasmic reticulum with little being detected in the Golgi apparatus which explains the retarded secretion.

C1q production by DCs raises the possibility of autocrine DC regulation by C1q. We then proceeded to study how C1q may influence DC development and found that C1q primed the development of DCs with tolerogenic properties. These C1q-conditioned DCs, which are expected *in vivo*, are better at clearing apoptotic cells, produce less inflammatory cytokines, and are less able to activate Th1 and Th17 cells. Higher ERK activity seems to contribute to these tolerance-related features of DCs differentiated with C1q. These properties suggest that the C1qDCs may raise the threshold of immune reactions or enhance tolerance, thus negating the development of SLE which inevitably involves the breakdown of self-tolerance.

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Abbreviations

7-AAD	7-Amino-actinomycin D
AC	apoptotic cell
APC	antigen presenting cell
BCS	bovine calf serum
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
C1q	complement component 1, subcomponent q
cDC	conventional dendritic cell
cDNA	complementary DNA
CLR	C-type lectin receptors
DC(s)	dendritic cell(s)
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCS	fetal calf serum
Fig.	Figure
FITC	fluorescein isothiocyanate
Fn	fibronectin
GM-CSF	granulocyte macrophage-colony stimulating factor
hi	high
hr	hour
IC	immune complex
IFN	interferon
Ig	immunoglobulin
IL	interleukin
int	intermediate
kDa	kilodalton
LDH	lactate dehydrogenase
lo	low
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MAPK	mitogen activated protein (MAP) kinase
mDC	human blood myeloid DC
MFI	mean fluorescent intensity
MHC	major histocompatibility class
min	minutes
moDC	human monocyte-derived dendritic cell
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor *kappa* B

NOS	nitric oxide synthase
OD	optical density
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PI	propidium iodide
PMA	phorbol 12-myristate-13-acetate
PRR(s)	pattern recognition receptor(s)
ROS	reactive oxygen species
RNA	ribonucleic acid
RPMI	RPMI-1640 culture medium
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	seconds
SLE	systemic lupus erythematosus
Syk	spleen tyrosine kinase
TBS	Tris-buffered saline
TCR	T cell receptor
TLR(s)	Toll-like receptor(s)
TNF	tumour necrosis factor
Tris	tri-hydroxymethyl-aminomethane

Chapter 1 Introduction

1.1 The immune system and its receptors

1.1.1 Innate and adaptive immunity

The immune system confers the ability for an organism to defend against exogenous microbial infection and also to respond to endogenously derived dangers such as malignancy and tissue damage. The vertebrate immune system is divided into two intimately linked arms, the innate and the adaptive immunity. The innate immune system reacts rapidly to dangers, possibly within hours or minutes, and in a general manner rather than specific to a particular pathogen or aberrant cell. It represents the first line of defense against microbial infections, including viruses, bacteria, fungi and parasites (Medzhitov and Janeway, 2000). In contrast, the adaptive immunity takes time to develop, about 4 – 7 days. It provides immunological memory, or lasting protection against re-encounters with a particular pathogen. Possibly, re-encounters with the specific antigen could result in an even stronger immune response against it.

The adaptive immune response comprises of T-cell mediated cellular immunity and B-cell mediated humoral or antibody immunity. T-cell and B-cell receptors are required for specific antigen recognition. An extremely diverse repertoire of B-cell and T-cell receptors are generated somatically during lymphocyte development because of the random nature of VDJ gene segment recombination during the

process of receptor gene rearrangement. Consequently, there is a high probability of the existence of an individual receptor on a single cell specific to a particular antigen. A lymphocyte with its receptor presented with its specific antigen by APCs would subsequently be activated and proliferates. The clonal selection and expansion of the destined cell is the key behind immunological response and immune memory in adaptive immunity.

The distinctive difference between the innate and adaptive immune systems lies in the receptors used for danger recognition. Innate immunity is mediated by germline-encoded receptors that have evolved to recognize a few highly conserved structures present in different groups of microorganisms, referred to as pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000). The receptors that recognize PAMPs are known as pattern recognition receptors (PRRs).

1.1.2 Pattern recognition receptors

Immune cells, particularly the antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs), express different PRRs that can be cell membrane-associated, in intracellular compartments or secreted into the blood stream and tissues, and all receptors facilitate the recognition of PAMPs. More recently, PRRs were discovered to also recognize endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Some PRRs capture pathogens and subsequently mediate their phagocytosis and endocytosis, and these are the phagocytic/endocytic receptors. Among the membrane-associated receptors of this category are the mannose receptor (MR), scavenger receptors (SRs) and

complement receptors (CRs) (Aderem and Underhill, 1999). There are also secreted PRRs or pattern recognition molecules (PRM) that could bind their targets and act as opsonins. These include the pentraxins (PTX) such as C-reactive protein (CRP), serum amyloid protein (SAP) and PTX3 (Gewurz *et al.*, 1995; Bottazzi *et al.*, 2006); collectins such as lung surfactant proteins A (SP-A) and D (SP-D) and mannose-binding lectin (MBL) (Kishore *et al.*, 2006; Takahashi *et al.*, 2006; Gupta and Surolia, 2007); complement components such as C1q (Lu *et al.*, 2008) and C3 (Sahu and Lambris, 2001); LPS-binding protein (LBP); and CD14 (Fenton and Golenbock, 1998; Schutt, 1999).

Sensing PRRs include the transmembrane Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). Cytosolic sensing PRRs include the RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs). Engagement of these receptors leads to signaling cascades resulting in transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells. However, aberrant activation of this system could lead to immunodeficiency, septic shock, or induction of autoimmunity (Takeuchi and Akira, 2010). Table 1.1 provides a summary of the ligands recognized by these sensing PRRs.

Currently, 10 TLRs are identified in humans and 12 in mice (Akira *et al.*, 2006). TLRs have extracellular N-terminal leucine-rich repeats, a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Stimulation of TLRs result in the recruitment of TIR domain-containing adaptors, such as MyD88 and TRIF, with downstream signaling cascades activating NF-κB, MAP

kinases and IRFs, leading to inflammation, as characterized by the production of cytokines, chemokines and type I interferon (Akira *et al.*, 2006).

The CLRs have one or more domains that are homologous to carbohydrate recognition domains and can exist both as soluble and transmembrane proteins (Geijtenbeek and Gringhuis, 2009). Some CLRs can induce signalling pathways that directly activate NF-κB, whereas other CLRs affect signaling by TLRs. DC expressed CLRs that have garnered interest lately include the DEC205, DC-SIGN, Dectin-1 and Dectin-2. Importantly, Dectin-1 and Dectin-2 are immunoreceptor tyrosine-based activation motif (ITAM)-coupled and are important for detection of β-glucans from fungi.DCs activated by Dectin-1 or Dectin-2 are shown to activate T cells and confer protective immunity against *C. albicans* (Robinson *et al.*, 2009)

RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain (Yoneyama and Fujita, 2008). They are cytoplasmic sensors that recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses. The CARDs of RLRs are activate the signaling cascades by interacting with the N-terminal CARD-containing adaptor IFN-β-promoter stimulator 1 (IPS-1) and the downstream signaling events activate type I interferon genes. NLRs are cytoplasmic pathogen sensors with a central nucleotide-binding domain and C-terminal leucine-rich repeats. The N-terminal harbor protein-binding motifs, such as CARDs, apyrin domain, and a baculovirus inhibitor of apoptosis protein repeat (BIR) domain (Takeuchi and Akira, 2010). TLRs and NODs can synergize and activate inflammatory cytokine production.

Table 1.1. PRRs and Their Ligands. Adapted from Takeuchi and Akira (2010).

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

1.2 Dendritic cells

1.2.1 Roles of DC in immunity and tolerance

DCs play a vital role in the immune system. As the major APC, DCs provide a bridge between innate and adaptive immunity (Banchereau and Steinman, 1998). In their immature state, DCs act as sentinels, and through its effective antigen sampling via macropinocytosis and endocytosis, they sense their macroenvironment for danger signals (PAMPs) from pathogens and endogenous sources (DAMPs). On sensing a danger signal, DCs undergo maturation, mount an immune response leading to inflammation and subsequent priming of the adaptive immunity. This is coupled to increased antigen processing and presentation by upregulation of both the MHC I and MHC II components, upregulation of costimulatory molecules such as CD40, CD80 and CD86 and increased cytokine production (eg. IL-12, IL-10, TNF-α and IL-6).

DCs that have undergone functional maturation would migrate to the T cell region of secondary lymphoid organs (Randolph *et al.*, 2005), and are highly efficient at stimulating T cells via 3 distinct signals, i.e. antigen specific TCR stimulation, costimulatory surface signals such as CD80/CD86 stimulation of CD28 receptor on T cells, and also cytokines such as IL-12. Internalized antigens are degraded, loaded onto MHC II complexes and presented to CD4 T helper cells that express the antigen-specific TCR. Endogenous antigens are processed and loaded onto MHC I for the priming of cytotoxic CD8 T cells that bear the specific TCR.

DCs can polarize adaptive immunity by inducing specific CD4 T helper cell subsets (Guermonprez *et al.*, 2002). The 3 main subsets of T helper responses currently studied are the Th1, Th2 and Th17 responses. Differentiation of naïve CD4 T cells into Th1 largely requires IL-12p70 production by DCs (Trinchieri, 2003). Th1 cells produce IFN-γ and TNF-β, and Th1 immunity is generally acknowledged to protect against intracellular pathogens and tumors. Th2 cells produce IL-4, IL-5 and IL-13 and are responsible for promoting humoral immunity against parasites. IL-4 and OX40L are some known factors that direct Th2 polarization (Ito *et al.*, 2005). Th17 cells are more recently discovered and characterized. They produce IL-17A, IL-17F, and IL-22 and its differentiation is promoted by TGF-β, IL-6, IL-21 and IL-23 (Korn *et al.*, 2009). Functionally, Th17 cells are implicated in defense against extracellular pathogens such as fungi, and development of autoimmune and inflammatory diseases such as psoriasis and rheumatoid arthritis.

Unabrogated inflammation caused by unabated T cell activation is detrimental to systemic homeostatis and can lead to autoimmunity. The normal immune system produces a population of T cells, called regulatory T cells (Tregs) that are specialized for immune suppression. Tregs are important in the maintenance of peripheral immunological self-tolerance. They suppress effector T-cell proliferation and thus can actively downregulate the activation and/or proliferation of self-reactive T cells (Sakaguchi *et al.*, 2008). Naturally occurring Treg arise in the thymus, and T cell activation usually induces a population of Tregs, and they are characterized as $CD4^{+}CD25^{+}FoxP3^{+}$. In addition to Treg cells, there also exists DCs with tolerogenic properties that are crucial regulators of immunity (Morelli and Thomson, 2007). Immature DCs are long been known to be tolerogenic, and mature

tolerogenic DCs do not express the full armada of strong stimulatory signals. Tolerogenic DCs can present antigen to antigen-specific T cells, but provides inadequate co-stimulatory signals (or deliver net co-inhibitory signals) for effector T-cell activation and proliferation. This can result in T-cell death, T-cell anergy or regulatory T-cell expansion or generation. Thymic DCs can negatively select autoreactive $CD4^+CD8^+$ thymocytes and induce tolerance to self antigens (Brocker *et al.*, 1997). Thus, tolerogenic DCs have been shown to suppress autoimmune conditions (Menges *et al.*, 2002; Verginis *et al.*, 2005).

1.2.2 Heterogeneity of DCs

There are loosely two categories of DCs. In the periphery, Langerhan cells and dermal DCs act as sentinels for pathogens or peripheral self-antigen, then undergo maturation and migrate via the lymphatics toward draining lymphoid organs and these are categorized as migratory DCs (Wilson *et al.*, 2003). Lymphoid tissue resident DCs are found in all lymphoid organs of the mouse, including the spleen and draining lymph nodes. These cells are immature in the steady state, and are $CD11c^{hi}$ $CD45RA^{lo}$ MHC II^{int}, which can be further broken into two broad subsets; the $CD8^+$ conventional DC (cDC) and the $CD8^-$ cDC (Naik, 2008). On maturation, these DCs become migratory and are MHC II^{hi}.

Plasmacytoid DCs (pDC) are $CD11c^{int}$ cells, and are considered as pre-DCs. At resting state, they resemble plasma B cells (Liu, 2005). In the steady state, pDCs express low levels of MHC I, MHC II and co-stimulatory molecules, and all are upregulated upon activation. Following activation, pDCs produce high levels of

type I interferon, and concomitantly acquire DC morphology and functions, such as antigen presentation and T cell activation.

In the human blood, DCs are a heterogeneous cell population originating from bone marrow precursors and they make up approximately 1% of circulating peripheral blood mononuclear cells (PBMCs) (Kassianos *et al.*, 2010). CD11c divides $lin^{-}HLA\text{-}DR^{+}$ blood DC into the $CD11c^{-}$ plasmacytoid (pDC) and $CD11c^{+}$ myeloid (mDC) subsets. pDCs represent about 18% of the blood DC population and is distinguished from mDC by their expression of CD123, CD303 (BDCA-2) and CD304 (BDCA-4/neuropilin-1). mDC comprises over 70% of blood DCs and can be subdivided into 3 subsets. The $CD1c^{+}$ (BDCA-1) subset makes up around 19% of blood DCs and is the most extensively studied mDC subset. The $CD16^{+}$ subset constitutes about 50% of blood DCs and has not been studied extensively due to CD16 depletion in many isolation protocols and their poor viability *in vitro*. The $CD141^{+}$ (BDCA-3) subset is the rarest, constituting around only 3% of blood DCs and is the least studied.

Due to the rarity of DCs in peripheral blood, in vitro experiments have mostly relied on DCs generated from mouse bone marrow cells supplemented with GM-CSF (Inaba *et al.*, 1992) or from human blood monocytes cultured with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). A glaring disadvantage of this method is DCs generated this way are highly inflammatory and are only found in vivo following an inflammation (Shortman and Naik, 2007). Thus, they do not represent the steady state DCs that represent the normal population of DCs in healthy individuals.

1.3 Systemic Lupus Erythematosus (SLE)

1.3.1 General overview

Systemic lupus erythematosus (SLE) or lupus is a multi-factorial systemic autoimmune disease affecting multiple organs, including the heart, joints, skin, lungs, blood vessels, liver, kidneys, and nervous system. The clinical presentations of the disease range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis (Rahman and Isenberg, 2008). SLE patients have genetic susceptibility and it predominantly affects women, especially those of reproductive age. Females of African American or Hispanic American origins have a 3–4 times increased risk of developing disease compared to Caucasians (Reveille *et al.*, 1998).

The underlying pathogenic mechanisms of SLE remain poorly understood and as a result, treatment options are limited. However, major progresses have been made in the understanding of this disease (Croker and Kimberly, 2005). The development of antinuclear antibodies is a hallmark in SLE. These antibodies form immune complexes (IC) with nuclear antigens (e.g. chromatin and RNP) and cause unabated type I IFN production from plasmacytoid DCs which is highly pathogenic in SLE (Banchereau and Pascual, 2006; Pascual *et al.*, 2006). These ICs can form or deposit in connective tissues to cause C1q-mediated complement activation leading to tissue inflammation and damages (Fliermann and Daha, 2007). Therefore, both type I IFN and C1q are in theory predicted to have detrimental roles in SLE development. However, hereditary C1q deficiency is strongly associated with SLE development which, in contrary, suggests a strongly protective role for C1q (Petry

and Loos, 2005). A connection between C1q and type I IFN in SLE has not been established which is expected to provide novel insights into the pathogenesis of this disease.

1.3.2 Antinuclear antibodies are characteristic of SLE and are pathogenic

In SLE patients, many autoantibodies develop. The mechanisms by which self-tolerance is broken down and hence allowing these autoantibodies to develop are unclear. Autoantibodies against nuclear antigens, such as chromatin/nucleosomes and ribonucleoproteins can form immune complexes (IC) with these autoantigens. IC are pathogenic in SLE (Banchereau and Pascual, 2006; Kyogoku and Tsuchiya, 2007). For instance, ICs that are deposited in the kidneys can cause glomerular nephritis.

Briefly, these IC can: **1**) activate plasmacytoid dendritic cells (pDC) to produce IFN-α. As discussed later, unabated IFN-α production is highly pathogenic in SLE. **2**) These IC can be captured by conventional DC and chromatin and RNP antigens can be presented by DCs to stimulate autoreactive T and B cells. This will cause persistent production of pathogenic autoantibodies. **3**) These IC can stimulate autoreactive B cells directly to produce anti-chromatin and anti-RNP autoantibodies.

1.3.3 Recent identification of type I interferon (IFN) in SLE pathogenesis

Beside complement, another major innate immune mechanism that is highly relevant to SLE pathogenesis is type I IFN, and in humans these include IFN-α and

IFN-β. Type 1 IFN was originally associated with conferring an antiviral response to cells before their roles in the autoimmune disease SLE were established by Pascual and Banchereau (Banchereau and Pascual, 2006; Pascual *et al.*, 2006). They signal through the IFN-α/β receptor that consists of two subunits IFNAR1 and IFNAR2. The intracellular domains of both subunits are associated with the Jak kinases Tyk2 and Jak1 respectively. IFN-α/β binding to the receptors activates both the Jak kinases, which leads to the phosphorylation of IFNAR1, Stat1 and Stat2. Stat1 could homodimerize to form the IFN-α-activated factor (AAF) or IFN-stimulated gene factor 3 (ISGF3) which is a heterotrimeric complex of Stat1, Stat2 and IRF9 (Honda *et al.*, 2005). Both complexes translocate into the nucleus, in which AAF binds to gamma-interferon activated sites (GAS) while ISGF3 binds to IFN-stimulated response element (ISRE). IFN-γ is the only member in the type II interferon family and it signals through the IFNGR1 and IFNGR2 receptor complex which is formed on ligand binding. It similarly leads to the transactivation of Jak1 and Jak2, phosphyrylation of IFNGR1, homodimerization and phosphorylation of Stat1 and finally its translocation to the nucleus (Ikeda *et al.*, 2002). Stat1 homodimers in the nucleus bind to specific GAS elements and thereby effect the transcription of IFNγ-induced genes.

Persistent production of IFN-α has been detected in SLE together with chronic expression of many IFN-regulated genes (Banchereau and Pascual, 2006; Kyogoku and Tsuchiya, 2007). Association of IFN-α with SLE was first indicated in 1979 when elevated serum IFN-α was found in SLE patients (Hooks *et al.*, 1979). Strong evidence that type I IFN is detrimental to SLE development came from therapeutic IFN-α treatment of cancer and viral infections because some patients treated with

IFN-α developed antinuclear antibodies, autoimmunity or even SLE (Ronnblom *et al.*, 1991; Kalkner *et al.*, 1998). More evidence is also supplied from recent genomics and proteomics studies in which the expression of IFN-regulated genes, known as the IFN signature genes, were found to be globally elevated in peripheral blood mononuclear cells and sera of SLE patients (Baechler *et al.*, 2003; Bennett *et al.*, 2003; Bauer *et al.*, 2006). Additionally, single nucleotide polymorphism studies revealed that TYK2 and IRF5, two main transcription factors in type I IFN signaling and production, are associated with SLE (Sigurdsson *et al.*, 2005).

Based on these data, prevailing models of SLE pathogenesis have been developed (Pascual *et al.*, 2006; Kyogoku and Tsuchiya, 2007; Pascual *et al.*, 2008). Briefly, pDCs produce high IFN-α upon infection with virus and other pathogens (e.g. EBV) and this type of IFN-α production normally subsides after infection is resolved. However, when autoreactive B and T cells specific for chromatin or RNP are activated, e.g. due to molecular mimicry or breach of tolerance, antibodies are produced that react with the autoantigens to form pathogenic IC, which further activates pDCs to produce more IFN-α. Pathogenecity of IFN-α in SLE is mediated by differentiating monocytes into inflammatory DCs, activation of DCs, activation of cytotoxic CD8 T cells to lyse cells and produce more autoantigens, and conversion mature B-cells into antibody producing plasma cells.

1.4 C1q

1.4.1 Structure of C1q

A C1q macromolecule consists of 18 polypeptide chains (6 A-, 6 B-, and 6 C-chains) (Fig 1.1). Each complete C1q polypeptide has a collagenous N-terminal half and a globular C-terminal half with the collagenous region from each of the three chain types forming a triple helix bringing the 3 C-terminal globules together (Reid and Porter, 1976). A C1q consists of 6 such heterotrimeric structures which are held together through inter-chain disulphide bonds at the N-terminal ends. It is viewed as a "bundle-of-tulips" under the electron microscope (Knobel *et al.*, 1975). At the genomic context, the three human C1q genes are clustered together in a stretch of chromosome 1, in the sequence of C1qA - C1qC - C1qB, and all in the 5' to 3' orientation (Sellar *et al.*, 1991).

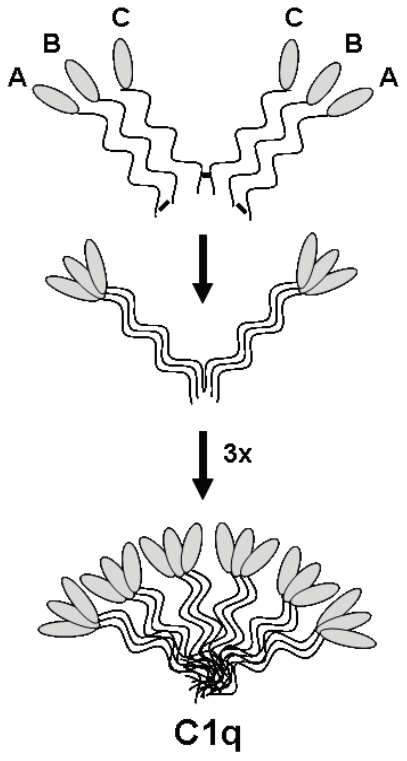

Figure 1.1. Assembly of the 18 polypeptide chains to form the C1q molecule. C1q is assembled in macrophages and DCs from three types of chains, i.e., A-chain, B-chain and C-chain. Each chain has a collagenous N-terminal half and a non-collagenous C-terminal half which form globules. A and B chains dimerize through a disulphide bond at the N-terminal end and two C chains form homodimers through similar disulphide bonding. An A-B dimer and a single C-chain form a triple helix and the other C-chain in a C-C dimer trimerizes with another A-B dimer forming to triple helices linked by the disulphide between the two C-chains. Three such structures form a C1q molecule through N-terminal association. Reproduced from (Lu *et al.*, 2008)

1.4.2 The classical roles of C1q and the complement system

1.4.2.1 The complement pathways

The complement system is an effector mechanism of the innate immune system, with 3 major roles in the defense against bacterial infection, bridging innate and adaptive immunity, and clearing inflammatory immune complexes and other inflammatory mediators following an inflammation (Walport, 2001). It is a complex interplay of 30-40 soluble plasma and cell surface proteins that proceeds through a series of proteolytic and complex-forming biochemical steps and culminate in the target destruction.

Three routes of complement activation are recognized: the classical (antibody-mediated) pathway, the mannose binding lectin (MBL) pathway, and the alternative pathway. Although each pathway has a unique combination of initiating proteins, all three converge in the activation of the complement component C3 and a common lytic pathway involving the formation of the membrane attack complex (MAC) on the target cell surface. The various complement pathways are schematically represented and described in Figure 1.2.

1.4.2.2 C1q in complement-mediated inflammation and defense against pathogens

C1q initiates the complement classical pathway by binding to the Fc portion of antibodies that bound to antigens on the surface of a bacterial cell (Duncan and Winter, 1988), and recruits C1r and C1s to form the C1 complex (one molecule of

C1q, two molecules of C1r, and two molecules of C1s) (Reid, 1986; Duncan and Winter, 1988). Complement activation can increase tissue inflammation through the generation of proteolytic complement fragments such as C3a and C5a which are anaphylatoxins to stimulate neutrophil chemotaxis to the affected tissue sites (Gasque, 2004). Another effector function of complement is the deposition of C3 and C4 fragments on the reacted surfaces (microbial or endogenous) and these fragments have receptors on phagocytes such as macrophages and neutrophils. As a result, it causes enhanced phagocytosis of complement reacted targets for clearance (Underhill and Ozinsky, 2002).

Complement can be activated by many microbial surfaces and the importance of this mechanism in host protection against microbial infections has been testified by the increased susceptibility of $C1q^{-/-}$ mice to microbial infections. With C1q deficiency, mice are more susceptible to reinfection by *Plasmodium chabaudi* parasites (Taylor *et al.*, 2001). These knockout mice also show increased infection upon multiple microbial challenges in the peritoneal cavity and increased susceptibility to *Salmonella enterica serovar Typhimurium* infection (Celik *et al.*, 2001; Warren *et al.*, 2002).

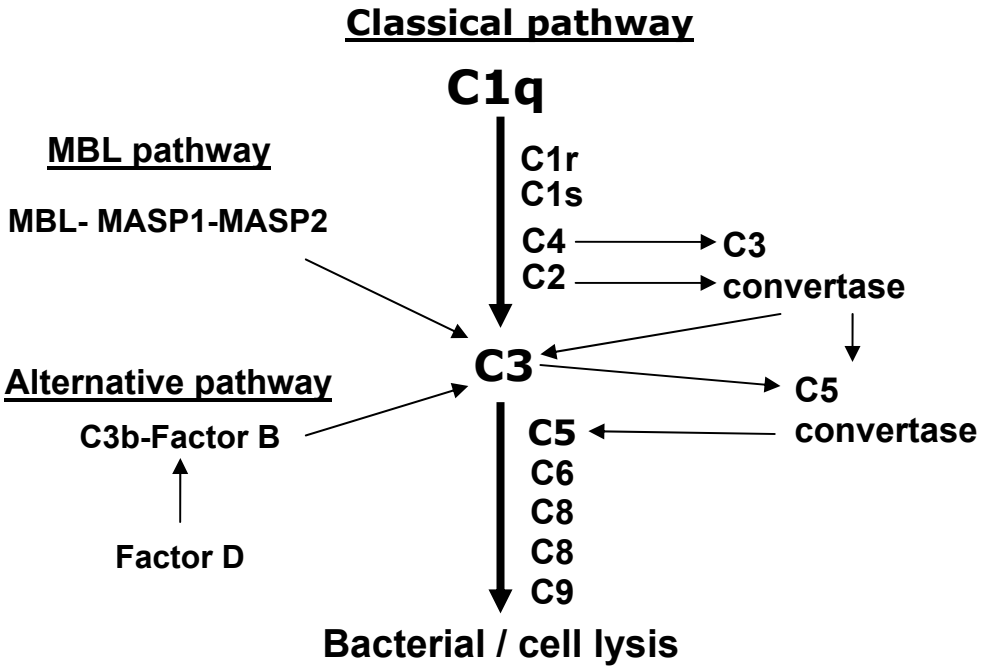

Figure 1.2. Schematic of the 3 pathways of complement activation - the Classical, Mannose-Binding Lectin (MBL), and Alternative Pathways. The three pathways converge at the point of cleavage of C3. The classical pathway is initiated by the binding of the C1 complex consisting of 1 C1q, 2 C1r and 2 C1s to antibodies bound to the bacterial cell surface. C1s first cleaves C4 and then cleaves C2, leading to the formation of a C4b2a enzyme complex (classical pathway C3 convertase). The MBL pathway is initiated by binding of the complex of MBL and MASP1and MASP2 to arrays of mannose groups on the bacterial cell surface. MASP2 acts as a protease like C1s, and facilitate classical pathway C3 convertase formation. The alternative pathway is initiated by the covalent binding of a small amount of C3b to hydroxyl groups on cell-surface carbohydrates and proteins and is activated by low-grade cleavage of C3 in plasma. This C3b binds factor B, a protein homologous to C2, to form a C3bB complex. Factor D cleaves factor B bound to C3b to form the alternative pathway C3 convertase C3bBb. The C3 convertase enzymes cleave many molecules of C3 to C3b, which bind covalently around the site of complement activation. Some of this C3b binds to C4b2a and C3bBb to form C5 convertase enzymes which cleave C5 into C5a and C5b. C5b recruits C6, C7, C8 and several C9 to form the MAC. Adapted from Walport, 2001

1.4.3 Other roles of C1q

In addition to the role of C1q in activating the complement classical pathway, numerous new studies have suggested additional physiological functions for this molecule. C1q was found to have chemotactic properties and stimulated migration for eosinophils (Kuna *et al.*, 1996), mast cells (Ghebrehiwet *et al.*, 1995), neutrophils (Leigh *et al.*, 1998) and fibroblasts (Oiki and Okada, 1988).

In addition to microbial killing via complement activation, C1q can directly bind to *Listeria monocytogenes* and this opsonizes the bacteria for enhanced macrophage uptake. C1q opsonized *Staphylococcus aureus* induces respiratory burst in neutrophils that is important for pathogen killing (Eggleton *et al.*, 1994). $C1q^{-/-}$ mice was significantly poorer in the uptake and cross-presentation of ova antigen-IC by DCs to $CD8^{+}$ T cells, and exogenously added C1q restored this deficiency (van Montfoort *et al.*, 2007). The $C1q^{-/-}$ mice also showed reduced ability to induce the proliferation and Th1 differentiation of antigen specific T cells (Baruah *et al.*, 2009) and its antigen specific T cells also produced less IFN-γ (Cutler *et al.*, 1998). Of notable interest in SLE pathogenesis, C1q was also found to enhance the clearance of apoptotic and secondary necrotic cells (Quartier *et al.*, 2005; Gullstrand *et al.*, 2009).

Several reports indicated that C1q can regulate cytokine production in different cells. C1q inhibited IL-4 and but enhanced IL-10 production by T cells (Lu *et al.*, 2007). In Lipid A-activated macrophages, autocrine C1q production stimulated TNF receptor synthesis, promoted TNF-α binding to the receptor and this induced nitric oxide synthase (NOS) synthesis (Jiang *et al.*, 1996). C1q added to human

umbilical vein endothelial cells stimulated the production of IL-6, IL-8 and MCP-1 (van den Berg *et al.*, 1998). C1q modulated cytokine production by DCs and could suppress inflammation leading to autoimmune conditions such as SLE (Yamada *et al.*, 2004; Csomor *et al.*, 2007).

In transmissible spongiform encephalopathies (TSEs), C1q had detrimental effects whereby mice deficient in C1q were highly resistant to scrapie isoform of prion protein (PrP^{Sc}) (Klein *et al.*, 2001) and had a delayed onset of disease (Mabbott *et al.*, 2001). It was further showed that PrP^{Sc} interacted directly with C1q to activate the classical complement pathway (Mitchell *et al.*, 2007). C1q is also an important pathogenic factor in Alzheimer's disease. In the brain, C1q binds to amyloid-β-peptide (Aβ) which is a major component of senile plaque, and this activates the complement system and ultimately result in inflammation and neurodegeneration (Shen and Meri, 2003). This pathogenic role of C1q is also observed in another related disease known as familial dementia (Bonifati and Kishore, 2007).

A recent paper indicated a novel role of C1q that is independent of complement activation, in phosphorylation and activation of the tumor suppressor WOX1 and causing its translocation into the nucleus (Hong *et al.*, 2009). This led to the destabilization of tumor cell adhesion and leading to apoptotic cell death. Another novel role of C1q that has emerged is its involvement in neuronal development and remodeling (Stevens *et al.*, 2007). In this model, C1q is hypothesized to be released from appropriately connected retinal ganglion cells, binding to neighbouring weaker synapses and targeting them for phagocytic removal.

1.4.4 C1q production and localization *in vivo*

1.4.4.1 C1q production is distinct from other complement components

Liver hepatocytes synthesize most of the complement proteins (Colten *et al.*, 1986). C1q is different from most complement proteins in that it is not synthesized by liver hepatocytes, but is was long recognized to be produced by tissue macrophages (Muller *et al.*, 1978; Loos *et al.*, 1989). C1q expression in DCs was first detected in follicular DC and interdigitating DCs in the spleen (Schwaeble *et al.*, 1995). In tonsil sections, DCs were also found to express C1q (Castellano *et al.*, 2004). More recently, C1q expression was shown in human bone marrow stromal macrophages and DCs (Tripodo *et al.*, 2007). Some reports have described C1q expression in other cell types such as endothelial cells (Cao *et al.*, 2003; Bulla *et al.*, 2008).

However Petry *et al*. (2001) showed *in vivo* that physiological C1q are of hemapoietic origins. They showed that, when wild type mouse bone marrow was transferred to irradiated $C1q^{-/-}$ mice, serum C1q levels and C1 function were rapidly restored and reached the levels of normal mice within 6 weeks of transplantation (Petry *et al.*, 2001). In reverse, when irradiated wild type mice received bone marrow from $C1q^{-/-}$ mice, serum C1q levels decreased over time and these mice became C1q deficient in 55 weeks.

1.4.4.2 C1q is found to deposit around tissue macrophages and DCs

C1q is abundantly present in the plasma, where healthy individuals have been reported to have C1q concentrations of 50 – 250 μg/ml (Dillon *et al.*, 2009).

Besides circulating in plasma, we previously discovered by immunohistochemical staining of arterial walls that C1q also deposited in tissues around DCs and macrophages, and both cells were expressing C1q (Figs. 1.3 and 1.4) (Cao *et al.*, 2003). Staining of biopsy specimens from patients with Barrett's esophagus (an inflammatory pre-malignant condition due to reflux of gastric contents), esophageal carcinoma or normal esophagus showed that DCs and macrophages expressed C1q, and C1q was again found surrounding DCs in the ECM (Bobryshev *et al.*, 2010).

How C1q deposits onto the tissue is unclear but it is structurally similar to the hexagonal type VIII and X collagens (Sellar *et al.*, 1991). It has been reported C1q interacts with extracellular matrix (ECM) molecules such as collagen, fibronectin and laminin (Menzel *et al.*, 1981; Bing *et al.*, 1982; Pearlstein *et al.*, 1982; Bohnsack *et al.*, 1985) and interactions with these common ECM proteins may aid in C1q deposition in the tissues.

Both macrophages and DCs are potent APCs and the deposited C1q around these cells may regulate their functions. For example, tissue deposition of C1q in the extracellular matrix may enable it to engage low affinity receptors on macrophages and DCs which are otherwise not activated by soluble C1q.

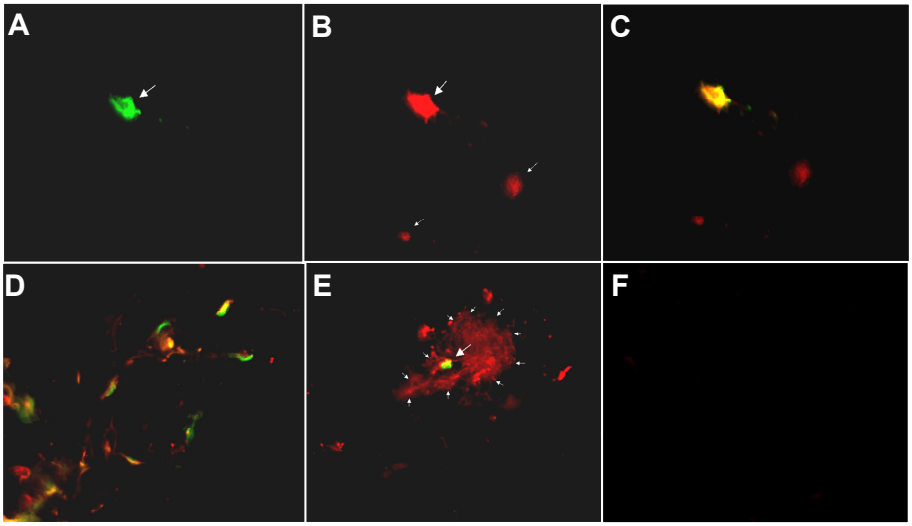

Figure 1.3. C1q is found inside and around DCs. Atherosclerotic lesions in arterial wall tissue sections were stained with DC-specific S-100 (green) and goat anti-C1q (red) antibodies. **(A–C)** A single DC in the intima (shown by a large arrow) expresses C1q **(C)** is a merged image of (A) and (B); **(B)** some other type cells (small arrows) also express C1q. **(D)** shows an area under the necrotic core of an atherosclerotic lesion containing several DCs and only some DCs express C1q. **(E)** C1q is present not only intracellularly in DC (large arrow, yellow cell), but also extracellularly (marked by small arrows). **(F)** Negative control. Reproduced from Cao et al (2003).

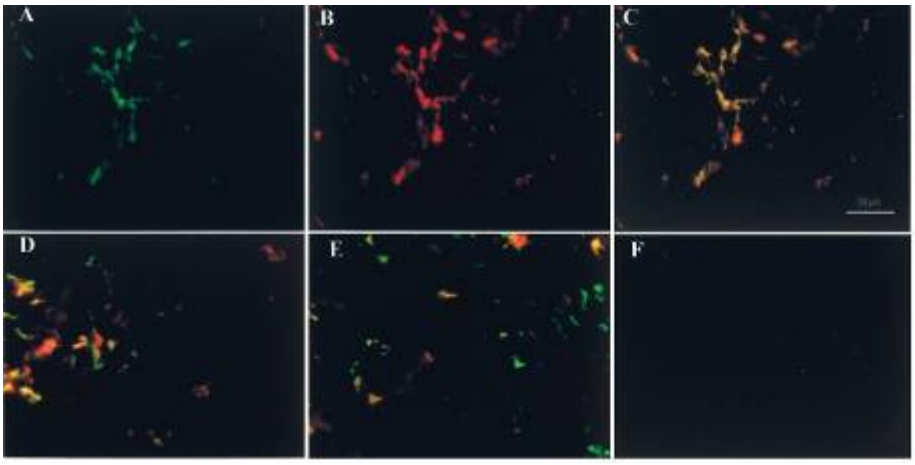

Figure 1.4. C1q is found inside and around macrophages. Expression of C1q (red) by macrophages (identified with anti-CD68, red) around the necrotic core in atherosclerotic lesions. **(C)** is a merged image of (A) and (B). **(D)** and **(E)** are merged images of two other areas in the atherosclerotic lesion. In C, D and E, note that the proportions of C1q expressing cells markedly vary. Around some C1q-expressing macrophages, C1q is also seen within the extracellular matrix. **(F)** negative control. Reproduced from Cao et al (2003).

1.4.5 The protein secretion pathway - how is C1q secreted?

1.4.5.1 The classical protein secretion pathway

Secreted proteins are mainly exported from mammalian cells by the classical endoplasmic reticulum/Golgi-dependent secretory pathway (Nickel, 2005). Sorting of the proteins into the correct compartments rely on specific signal tags on the protein itself. In the cytosol, newly synthesized nascent polypeptide chains emerging from ribosomal complexes are targeted into the endoplasmic reticulum (ER), where it undergoes chaperone-assisted folding, glycosylation and quality control steps to direct misfolded proteins for degradation (van Vliet *et al.*, 2003). They then exit the ER at specialized membrane domains called ER exit sites or tER sites, are then packaged into transport vesicles containing coat protein complex II (COPII)-coat. The COPII vesicles fuse to become the ER-Golgi intermediate compartment (ERGIC). Many ERGICs merge to form the *cis*-Golgi network (CGN). Secretory proteins are then further modified, processed and transported across the Golgi cisternae to the *trans*-Golgi network (TGN), or Golgi exit site. Here, final modifications are performed on the proteins before they are sorted, packaged and dispatched towards their final destination. These could be directed towards the apical and basolateral membranes in polarised cells, regulated secretory granules, the endosome/lysosome system or retrograde transported back to earlier compartments of the pathway (van Vliet *et al.*, 2003).

1.4.5.2 Unconventional protein secretory routes

Recently, some proteins have been shown to be secreted independent of the ER and Golgi compartments of the classical secretory pathway (Nickel and Rabouille, 2009). These unconventional protein secretions happens for proteins that have signal-peptides that are targeted to the ER, but are secreted independent of the Golgi network, or some proteins that lack signal peptides exit the cell independent of both the ER and Golgi pathways.

1.4.5.3 How is C1q processed and secreted?

A review of the C1q literature revealed that no study has been performed to investigate how C1q is secreted. It is difficult to imagine that C1q could be secreted independent of the ER/Golgi system of protein synthesis and secretion. The dimerization of the A-B and C-C chains requires correct disulplide bond formation, which usually occurs in the ER for eukaryotes. This involves protein disulphide isomerases (PDI) that works in concert with chaperones that can also perform checks to ensure correct disulphide bond formation (Fomenko and Gladyshev, 2003). Further assembly of the 6 A-B and C-C chains to give the complete C1q macromolecule inconceivably requires chaperone assistance in the ER. The failure of proteins to fold and assemble properly results in their retention in the ER and eventually leads to their degradation. The collagen domain of C1q was found to be hydroxylated at certain proline and lysine resides (Reid, 1974). Inhibiting the hydroxylation of proline and lysine residues blocked C1q synthesis in macrophages (Muller *et al.*, 1978; Mocharla *et al.*, 1987). In general, hydroxylations of proline and lysine residues are required for stabilization of the collagen triple helix

structure and some of the enzymes involved have been found in the ER, such as prolyl 4-hydroxylase (Gorres and Raines, 2010) and lysyl hydroxylase 3 (Myllyla *et al.*, 2007). This suggests that C1q synthesis, assembly and secretion require passage into the ER component of the classical secretory pathway.

1.4.6 Association of C1q deficiency with SLE

In addition to its classical role in complement activation and protection against microbial infection, C1q may also protect against autoimmunity. C1q deficiency in human is strongly associated with SLE-like autoimmune conditions, and C1q deficiency is the strongest genetic susceptibility to SLE, where more than 95% C1q-deficient human subjects develop SLE (Kolble and Reid, 1993). A protective role for C1q against SLE was strongly enhanced by the finding that $C1q^{-/-}$ mice also develop SLE-like conditions (Botto *et al.*, 1998).

1.4.6.1 Known mechanisms by which C1q may be connected to autoimmunity

C1q may reduce SLE through enhanced clearance of apoptotic cells (ACs). Excessive apoptotic cell exposure can cause autoimmunity (Mevorach *et al.*, 1998). Increased apoptotic body/chromatin levels have been detected in the blood of SLE patients (Decker, 2006). $C1q^{-/-}$ mice also showed increased tissue accumulation of apoptotic cells (Botto *et al.*, 1998). However, elevated cell death or increased circulating nucleosomes does not necessarily cause autoimmunity unless tolerance is breached (Holdenrieder *et al.*, 2001). The breach of self-tolerance is the key to SLE which originates ultimately from hyperactive APCs.

C1q was found to bind directly to surface blebs on ACs through calreticulin (Korb and Ahearn, 1997), and these opsonized cells are cleared by phagocytosis through CD91 (Ogden *et al.*, 2001). Another indirect role of C1q in AC clearance requires IgM binding to late ACs (Zwart *et al.*, 2004). C1q then binds IgM to activate the complement classical pathway, leading to C3 deposition and enhanced AC phagocytosis (Quartier *et al.*, 2005).

$C1q^{-/-}$ mice could develop autoantibodies by increased positive selection for autoreactive B1 cells by intracellular self antigens, and decreased negative selection of autoreactive conventional B cells by the same antigen (Ferry *et al.*, 2007). Possibly, C1q plays a role in the negative selection of the autoreactive B cells in normal mice, and defective early B cell development checkpoints resulting in autoantibody production have been reported in SLE patients (Yurasov *et al.*, 2005).

C1q was also reported to inhibit the production of inflammatory cytokines. It was observed that $C1q^{-/-}$ mice produced more IL-12p40 than wild type mice when these mice were peritoneally injected with LPS (Yamada *et al.*, 2004). A more recent study showed that immobilized C1q directly inhibited the production of some pro-inflammatory cytokines while it enhanced the production of some anti-inflammatory mediators such as IL-10 and IL-1 receptor antagonist (Fraser *et al.*, 2006). The dual roles played by C1q in enhancing AC clearance and inhibition of inflammatory cytokines suggest an anti-inflammatory role for C1q which may protect against SLE.

1.4.6.2 The selective C1q production by macrophages and DC, especially the latter, may hold important answers to its protective role against SLE

Hereditary C1q deficiency is a very rare cause of SLE. More commonly, patients acquired partial deficiency of C1q due to complement activation and approximately one third of SLE patients develop anti-C1q antibodies and the detection of these antibodies strongly correlated to reduced serum C1q (Botto and Walport, 2002). Whether the production of C1q itself is reduced in SLE patients remains unclear and could be a novel mechanism in SLE development and pathogenesis.

The complement system consists of more than 20 proteins which are mostly produced in the liver by hepatocytes, but C1q production is restricted to APC and its production by DCs implies a role in the regulation of adaptive immunity and tolerance (Muller *et al.*, 1978; Cao *et al.*, 2003). Blood monocytes only produce C1q after they differentiate into DCs and macrophages (Lu *et al.*, 1996), although C1q is predominantly found in the blood circulation like monocytes. This mode of C1q production could subject DCs and macrophages to local autocrine or paracrine regulation by C1q under steady state. Immune complexes (IC) are a primary C1q ligand, which means that de novo C1q production by APC enables it to modify APC response to IC. For example, C1q binding to IC could modulate IC uptake by, and stimulation of, DCs to change the overall host immune response to these pathogenic identities. The extravascular tissue production of C1q also leaves a fraction of C1q deposited around these APC which may also regulate DC in T cell stimulation (Cao *et al.*, 2003; Csomor *et al.*, 2007). Overall, C1q production by APC, especially DCs, points to a potential role for C1q in the regulation of tolerance and IC processing. While recent studies have began to assess C1q in DC

regulation, how the DC origin of C1q is related to the known SLE pathogenic mechanisms has not been investigated.

1.4.7 How is C1q production by macrophages and DC regulated by microbial and SLE-relevant stimuli

1.4.7.1 Interferons

IFN-γ has been found to increase or inhibit C1q expression in different studies. Murine peritoneal macrophages stimulated with various concentrations of IFN-γ produced a dose-dependent increase in C1q mRNA and increased the extracellular accumulation of C1q (Zhou *et al.*, 1991a). A similar study using cultured mouse microglia found that IFN-γ also increased C1q mRNA (Haga *et al.*, 1996). A corresponding increase in C1q production following IFN-γ stimulation was also observed in human THP-1 derived macrophages and primary human monocyte-derived macrophages (Walker, 1998; Kaul and Loos, 2001). In another study, IFN-γ treatment of rat Kupffer cells which are liver tissue macrophages downregulated C1q production (Armbrust *et al.*, 1997). Curiously, $C1q^{-/-}$ mice show a reduced capacity to produce IFN-γ, suggesting that C1q and IFN-γ are engaged in a mutual immunoregulatory mechanism (Cutler *et al.*, 1998). How IFN-α affects C1q expression is yet to be determined and this may have implications in the pathogenesis of SLE.

1.4.7.2 TLR ligands

Microbial toll-like receptor (TLR) ligands can upregulate C1q production by macrophages or DCs (Zhou *et al.*, 1991b; Baruah *et al.*, 2006). A study using human monocyte-derived DCs showed that the TLR4 ligand, LPS can potently upregulate C1q production while two TLR2 ligands, LTA and PGN also enhanced C1q levels but to a lower extent (Baruah *et al.*, 2006). Peritoneal macrophages treated with C3b-opsonized zymosan also showed increased C1q production and secretion (Zhou *et al.*, 1991b). However, there are also reports of LPS being able to suppress C1q production. In the same study where IFN-γ treatment downregulated C1q production, the authors also showed that LPS can also suppress C1q production in Kupffer cells (Armbrust *et al.*, 1997). Another study showed that LPS maturation of human monocyte-derived DCs and DCs generated from CD34+ hematopoietic stem cells inhibited C1q production (Castellano *et al.*, 2004). The study using mouse microglia found no difference in the expression of C1q mRNA after LPS treatment, but the levels from untreated cells were also non-detectable prior to IFN-γ stimulation (Haga *et al.*, 1996).

1.4.7.3 Drugs

Phorbol 12-myristate 13-acetate (PMA) is an activator of protein kinase C and was shown to inhibit C1q production by THP-1 derived macrophages (Walker, 1998). The drug tacrine is a cholinesterase inhibitor used in the treatment of Alzheimer's disease. Tacrine inhibited C1q upregulation by IFN-γ in THP-1 derived macrophages (Zhou *et al.*, 1991b). Some non-steroidal anti-inflammatory drugs inhibited C1q production from thioglycollate-elicited macrophages but the same drugs upregulated C1q production from resting resident macrophages (Trinder *et al.*,

1995). The organic gold compound auranofin greatly enhanced C1q production at both transcriptional and secretory levels in thioglycollate-elicited macrophages whilst, conversely, AFN reduced mRNA levels in resident macrophages. It is interesting to note that C1q expression is generally upregulated under immunosuppressive conditions. For example, a microarray study revealed that tumor-associated macrophages expressed elevated levels of C1q and two major immunosuppressive cytokines IL-10 and TGF-β (Biswas *et al.*, 2006). Immunosuppresive drugs like dexamethasone, prednisone and hydrocortisone all enhanced C1q production in macrophages (Trinder *et al.*, 1995; Armbrust *et al.*, 1997; Walker, 1998).

1.4.7.4 Conclusion

The regulation of C1q production is likely to have major impacts on SLE development but the mechanisms are poorly understood. The discrepancy in conclusions from studies on regulation of C1q production may have cell type, species and methodical contributions. It would be useful to have a systematic study with a single system used to examine the modulator effects of SLE-relevant stimuli in C1q production.

1.5 Aims of this study

C1q has diverse functions on a myriad of cell types. The aim of this study is to study C1q in the context of SLE pathogenesis where it has pathophysiological relevance. With different disease models linking its deficiency to SLE, understanding the processes that could suppress its production and understanding how the lack of C1q affects immune cells may lead to better understanding of the disease.

The overall aims of this study are to:

(1) Identify factors that may affect C1q production by DCs

(2) Understand the mechanisms in which C1q production by DCs is regulated by certain factors

(3) Investigate whether surface-deposited C1q, which was found around DCs in vivo, can regulate DC differentiation and activation

(1) Identify factors that may affect C1q productionby DCs

SLE is a complex autoimmune disease which involves chronic inflammation in multiple organs causing wide-spread tissue damages (Tsokos and Kammer, 2000; Croker and Kimberly, 2005). Also, SLE pathogenesis has complex pathogenic contributions from environmental and genetic factors. The etiology of SLE remains poorly understood, and what are the environmental factors that cause SLE are unclear. Here we search for stimuli that downregulated the production of C1q which is relevant to SLE pathogenesis.

To this end, a wide range of microbial structures, inflammatory factors, hormones and drugs will be screened for their ability to increase or decrease C1q production in DCs which may potentially trigger SLE under certain circumstances.

(2) Understand the mechanisms by which C1q production by DCs is regulated by certain factors

The protective roles of C1q in SLE have been strongly documented. We hypothesize that downregulation of normal C1q production in DCs could be detrimental to the patient and may be another underlying cause of SLE. This assumed that the known C1q effector functions, e.g. clearance of apoptotic cells or pathogenic IC which are ligands of C1q, is critical to SLE prevention.

Factors that are identified after aim (1) and have known roles to play in SLE pathogenesis will be studied in further detail.

(3) Investigate whether surface-deposited C1q, which was found around DCs in vivo, can regulate DC differentiation and activation

We have previously detected C1q deposition in arterial wall tissues around DCs *in vivo* by immunohistochemistry (Cao *et al.*, 2003). How deposited C1q may functionally regulate DC development from monocytes has not been investigated and this will be carried out in this study.

Chapter 2 Materials and Methods

2.1 Cell Biology Techniques

2.1.1 Isolation of monocytes from human buffy coats

Enriched peripheral blood leukocytes were obtained from the National University Hospital (NUH) Blood Donation Centre, Singapore in the form of buffy coat preparations derived from healthy donors. The buffy coats were diluted two-fold in PBS. 30 ml of diluted buffy coat was layered on 12 ml of Ficoll-Paque (Amersham Bioscience Corp., Piscataway, NJ) in a 50 ml tube and centrifuged for 30 min at 400X g without brake. Peripheral blood mononuclear cells (PBMCs) were collected from the gradient interface, resuspended in 50 ml of PBS and pelleted down by centrifugation at 200X g for 15 min to remove platelets. This washing step was repeated once, followed by two washes at 100X g each for 10 min. The washed cells were resuspended in 60 ml of RPMI medium containing 5% (v/v) BCS, 100 units/ml penicillin and 100 μg/ml streptomycin and then divided into three T75 cell culture flasks and cultured for 2 hr at 37ºC.

Non-adherent cells, which are mainly lymphocytes, were removed by four washes with 10 ml warm RPMI containing the above-mentioned supplements. The adherent fraction, mainly monocytes, was harvested by gentle scraping. Monocytes isolated by this method were >90% pure based on the expression of CD14 as judged by flow cytometry.

2.1.2 *In vitro* culture of monocyte-derived dendritic cells and macrophages

The isolated monocytes were cultured in 6-well tissue culture plates at a density of 1.5 x 10^6/ml in a DC culture cocktail, which is RPMI containing 10% (v/v) BCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and 0.0012% (v/v) β-mercaptoethanol. The cells were cultured for 6 days in the presence of 20 ng/ml of GM-CSF (R & D Systems Inc, Minneapolis, MN) and 40 ng/ml of IL-4 (R & D Systems Inc) to differentiate the cells into DCs. To culture DCs on immobilized C1q (C1qDCs), culture plates were coated overnight at 4°C with C1q (Sigma-Aldrich, St Louis, MO) or as a control, with BSA (both at 50 μg/ml in PBS) and the coated wells were washed 2X with PBS before cells were seeded. Differentiation of monocytes to macrophages was performed by adding 20 ng/ml of M-CSF (R & D Systems Inc.) instead. Every other day, half volume of the culture medium was removed and replenished with fresh media containing the respective cytokines at the stated concentrations.

2.1.3 Culture of mouse bone marrow-derived DC (BMDC)

Primary mouse bone marrow-derived DC were obtained from the laboratory of Dr Wong Siew Heng, Department of Microbiology, National University of Singapore. Briefly, cells were flushed from the long bones and cultured in DMEM containing 10% FBS and 20 ng/ml GM-CSF (day 0). Cells were then fed with fresh complete DMEM containing 20, 15, and 10 ng/ml GM-CSF on day 2, 4, and 6, respectively. The cells were used on day 6 or 7 (Ho *et al.*, 2008).

2.1.4 Isolation and sorting of mouse splenic DC

Mouse $CD8^+$, $CD8^-$ and plasmacytoid DC (pDC) were obtained from the laboratory of Professor Ken Shortman, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. A summary of the protocol used for the isolation process is given (Vremec *et al.*, 2000) – Spleens were cut into small fragments and then digested for 25 min at room temperature in 10 ml RPMI 1640-FCS medium containing type II collagenase, DNase I and EDTA. Undigested fibrous material was removed by filtration. All subsequent steps were at 0–4°C in a balanced salt solution containing EDTA (EDTA-BSS). Cells were recovered from the digest by centrifugation, the pellet was resuspended in a 1.077 g/cm^3 isoosmotic Nycodenz medium and centrifuged at 1700X *g* for 15 min, and then the low-density fraction was collected. The low-density cells were diluted in EDTA-BSS, recovered by centrifugation, and then incubated for 30 min with the following mAb: anti-CD3 (KT3-1.1); anti-Thy 1 (T24/31.7, a pan-Thy 1); anti-Gr1 (RB68C5); and anti-erythrocyte (TER-119). The cells coated with mAb were removed using anti-rat Ig coupled magnetic beads in a 5:1 ratio. The splenic DC (around 85% pure) were suspended in EDTA-BSS for presorting, staining, and analysis or sorting.

2.1.5 Cell line culture

Raw264.7 and Jurkat cells were obtained from the American Type Culture collection (ATCC) (Rockville, MD). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) for Raw264.7 or RPMI 1640 medium for Jurkat cells. Either medium was supplemented with 10% bovine calf serum (BCS) (HyClone, Logan, UT), 2 mM Lglutamine, 1mM sodium pyruvate, 100 units/ml

penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C in the presence of 5% CO_2. The cells were sub-cultured every 3 days at a ratio 1:4. Raw264.7 cells are adherent cells and were removed by gentle scraping whereas Jurkat cells are suspension cells and removed by pipetting out the medium from the culture flask.

2.1.6 Stimulation of cells with various agents

Differentiated cells that were ready for use were harvested by gently scraping with a cell scraper and washed 2X. For all cell washings and culture, an activating medium consisting of RPMI 1640 supplemented with 10% heat inactivated BCS, 100 units/ml penicillin and 100 μg/ml streptomycin was used unless stated otherwise. Cell stimulations were mostly performed in triplicates in 96-well plates with 100 μl of cell suspension at a density of 0.7 X 10^6 cells/ml. For extraction of RNA and protein where higher number of cells were required, 1 X 10^6 cells were seeded per stimulation in 2 ml of medium in a 6-well plate.

PAMPs and stimuli

The pathogen derived stimuli, cytokines/chemokines, drugs and hormones with the common dosage used to treat cells in this study are listed in Tables 2.1 to 2.3.

Table 2.1. PAMPs used in this study.

Pathogen derived stimuli	Source	Receptors targeted	Final concentration
Peptidoglycan (PGN)	Invivogen	TLR2, NOD1, NOD2	10 μg/ml
Pam3CSK4	Invivogen	TLR2, TLR1	5 μg/ml
Lipoteichoic acid (LTA)	Invivogen	TLR2	10 μg/ml

Zymosan	Invivogen	TLR2, Dectin-1	50 μg/ml
FSL-1	Invivogen	TLR2, TLR6	1 μg/ml
Pam2CSK4	Invivogen	TLR2, TLR6	0.1 μg/ml
Poly(I:C)	Invivogen	TLR3	50 μg/ml
Lipopolysaccharide (LPS)	Sigma	TLR4	0.5 μg/ml
Flagellin (Fla)	Invivogen	TLR5	1 μg/ml
CL097	Invivogen	TLR7/8	1 μg/ml
CpG oligonucleotide type A (CpGA)	Invivogen	TLR9	2 μM
CpG oligonucleotide type B (CpGB)	Invivogen	TLR9	2 μM
CpG oligonucleotide type C (CpGC)	Invivogen	TLR9	2 μM
TriDAP	Invivogen	NOD1	10 μg/ml
Muramyl dipeptide (MDP)	Invivogen	NOD2	10 μg/ml
Curdlan	Sigma	Dectin-1	50 μg/ml
Depleted zymosan	Invivogen	Dectin-1	50 μg/ml

Table 2.2. Cytokines and chemokines used in this study.

Cytokines / chemokines	Source	Final concentration
BAFF	Peprotech	0.1 μg/ml
soluble CD40 ligand (sCD40L)	R & D	1 μg/ml
fMLP	Invivogen	1 μM
GM-CSF	R & D	20 ng/ml
IFN-α	PBL	200 U/ml
IFN-γ	R & D	0.1 μg/ml
IL-1β	R & D	0.1 μg/ml
IL-3	Peprotech	10 ng/ml
IL-4	R & D	40 ng/ml
IL-6	R & D	0.1 μg/ml
IL-10	R & D	0.05 μg/ml
M-CSF	R & D	20 ng/ml
MCP1	R & D	0.05 μg/ml
Osteoprotegerin (OPG)	R & D	0.5 μg/ml
TGF- β	R & D	2 ng/ml
Thymic stromal lymphopoietin (TSLP)	R & D	15 ng/ml
TNF-α	Peprotech	0.05 μg/ml

Table 2.3. Drugs and hormones used in this study.

Cytokines / chemokines	Source	Final concentration
Cortisone	Sigma	1 μM
Dexamethasone	Sigma	1 μM
Hydrocortisone	Sigma	10 μM
Prednisone	Sigma	1 μM
Estradiol	Sigma	20 μg/ml
Progesterone	Sigma	20 μg/ml
Human chorionic gonadotropin (HCG)	Sigma	200 U/ml
Insulin	Sigma	100 nM
Leptin	Sigma	10 nM

Pharmacological inhibitors

In certain experiments, cells were treated with pharmacological inhibitors before further stimulation. In these cases, cells were pre-treated with the indicated inhibitor at specified dosages for 2 hr in a 37°C CO_2 incubator. All inhibitors were purchased from Calbiochem. These inhibitors usually come in powdered form and are typically dissolved in DMSO for storage. A detailed list of the inhibitors used is given in Table 2.3

Table 2.4. Pharmacological inhibitors used in this study.

Inhibitor	Target	Stock concentration	Working concentration
Wortmannin	phosphatidylinositol 3-kinase	100uM	5 nM
LY 294002	phosphatidylinositol 3-kinase	100mM	25 μM
Rapamycin	mTOR	200 μM	20 nM
Akt Inhibitor VIII	Akt	50 mM	40 μM
SC23766	Rac1	100 mM	50 μM
JNK Inhibitor II	*c*-Jun N-terminal kinase	50 mM	20 μM
PD98059	ERK	50 mM	40 μM
U0126	MAP kinase & Mek inhibitor	50 mM	10 μM

Chelerythrine chloride	Protein kinase C	12.5 mM	0.5 μM
SB 203580	p38 kinase	50 mM	40 μM
GW 5074	Raf1 kinase	20 mM	1 μM
Piceatannol	Syk kinase	41mM	25 μM
BAPTA-AM	Ca_{2+} influx		30 μM
Diphenyleneiodonium chloride (DPI)	iNOS, NADPH oxidase	5 mg/ml	1 μM
Pyrrolidine-2	cPLA2α	2 mg/ml	10 μM

2.1.7 Total, naïve and memory $CD4^+$ T cell isolation

Non-adherent PBMCs following monocyte isolation, were either processed fresh for T cell isolation, or stored at 4ºC overnight in RPMI supplemented with 20% BCS for T cell isolation the next day. The following kits from Miltenyi Biotec (Germany) were used for isolation of the respective cells:– $CD4^+$ T cell isolation kit II, naïve $CD4^+$ T cell isolation kit and memory $CD4^+$ T cell isolation kit. Cells were negatively selected using these kits, i.e. they were not labeled with antibody-conjugated magnetic beads. The kits were used following manufacturer's instructions. Briefly, total cell number was first determined and then resuspended at 10^7 cells per ml in MACS buffer. Cells were centrifuged at 600X g for 5 mins. Supernatant was removed and the cell pellet was resuspended in MACS running buffer at 40 μl per 10^7 cells. 10 μl of biotinylated antibodies, which are specific for cells other than the cells of interest, was added per 10^7 cells, mixed well and incubated on ice for 10 mins. 30 μl of MACS buffer and 20 μl of anti-biotin antibody-conjugated microbeads were added to each 10^7 cells. These were incubated on ice for 15 mins. Cells were then washed by centrifugation and then resuspended in 500 μl MACS buffer. Cells were then separated by magnetic separation on the autoMACS Separator (Miltenyi Biotec, Germany) using the

'depletes' program. Isolated cells were then washed once in RPMI media and centrifuged at 600X g for 5 minutes. The purity of isolated cells was assessed based on surface CD3, CD4, CD45RO (memory cells) and CD45RA (naïve cells) expression.

2.1.8 Isolation of plasmacytoid DC and myeloid DC from PBMC

The Diamond plasmacytoid DC (pDC) Isolation Kit and human CD1c (BDCA-1)$^{+}$ myeloid DC (mDC) Isolation Kit from Miltenyi Biotec were used for the isolation of these two types of human DC and according to the manufacturer's instructions. Magnetic separation was performed using the autoMACS Separator. Only freshly obtained PBMCs, i.e. without prior monocyte adhesion, were used with these two kits. About 1-2 X 10^{8} cells were used per isolation due to the rarity of these cells in the peripheral blood. pDC purity was assessed by CD123 and CD303 (BDCA-2) double expression on the surface while mDC purity was determined by the phenotype of $CD19^{-}$,$CD1C^{+}$.

2.1.9 Cell adhesion assay

Surface adhesion of monocytes which were differentiating into DCs was examined in 96-well plates pre-coated with eithere PBS, or C1q (50 μg/ml), BSA (50 μg/ml) or poly-L-Lysine (50 μg/ml). The plates were coated overnight at 4ºC and washed. 200 μl of monocytes (0.5 X 10^{6} cells) in the DC cocktail culture containing 20 ng/ml of GM-CSF and 40 ng/ml of IL-4 were added to the wells in triplicates. Adherent cells were detected at days 1, 2, 4, 6 and 7 after washing the plates twice

with warm culture medium, and incubation for 10 min with 0.1% (w/v) crystal violet in 10% ethanol (v/v) in PBS. The plates were washed 4 times using warm PBS and crystal violet was released from the adherent cells using 2% SDS (50 µl per well). The absorbance was read at 570 nm. Alternatively, DCs cultured in normal and C1q-coated plates were resuspended at 5 X 10^5/ml and stimulated for 24 hr in 96-well plates with LPS, IFN-γ, or both. Adherent cells were then stained using crystal violet and bound dye was eluted and measured.

2.1.10 DC macropinocytosis

DCs were washed and incubated for 30 min at 37^{o}C with FITC-dextran (1 mg/ml) (Molecular Probes, OR) in normal culture medium supplemented with 25 mM HEPES buffer (pH7.4). As a control, DCs were also incubated with FITC-dextran for 30 min at 4^{o}C. After 30 min, the cells were immediately chilled with an excess volume of ice cold culture medium and centrifuged at 600X g for 5 min. Two further washes were performed and the cells were fixed with 1% (w/v) paraformaldehyde in PBS. The uptake of FITC-dextran was analyzed via flow cytometry.

2.1.11 Mixed Lymphocyte Reaction (MLR)

For MLR, 0.1 ml T cells (2x10^6/ml) were co-cultured for 7 days with 0.1 ml DCs or macrophages (2x10^5/ml) in activation medium, in the presence or absence of LPS/IFN-γ. The culture supernatant was collected and IFN-γ and IL-17 were measured by ELISA.

For intracellular cytokine staining, at day 7, the cells were treated with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) and ionomycin (1μg/ml) for 6 hr. Brefeldin A (10 μg/ml) was then added for the last 4 hr. Cells were processed using a Cytofix/Cytoperm Cell Fixation and Permeabilization kit (BD Biosciences). After staining with IL-17 and IFN-γ antibodies for 20 min on ice, the cells were analyzed by flow cytometry. DC stimulation of allogeneic naïve CD4 T cells was also assessed based on CD25 induction after MLR in which the cells were stained with CD4 (Pacific blue) and CD25 (PE) antibodies.

For the re-stimulation experiments, cells were washed at day 7 of MLR and re-stimulated for 2 days using beads coated with α-CD3/α-CD28 antibodies (bead:cell ratio = 2:1). IFN-γ and IL-17 production was determined by ELISA on culture supernatant. In similar experimental settings, the DC/naïve cell MLR was re-stimulated at day 7 with the beads to examine CD25 expression on the CD4 T cells.

2.1.12 Generation of anti-CD3 and anti-CD28 antibody latex beads

Antibody-coated beads were used at a ratio of 2 beads to 1 T cell, i.e. 4 X 10^5 beads per well. An appropriate volume of latex beads (Polybeads microsphere 2μm, Polysciences Inc, USA) was resuspended in 1 ml PBS and centrifuged at 14 000X g for 5 minutes. Supernatant was removed and beads were resuspended in 1 ul PBS per 2 X 10^6 beads. 0.1 μg anti-human CD3 and 0.1 μg anti-human CD28 (Ancell, MN USA) were used to coat 4 X 10^5 beads by incubation in a 37°C CO_2 incubator for 2 hr. Beads were then resuspended in 1 ml activation medium and centrifuged at

14,000X g for 5 minutes. Supernatant was removed and beads were resuspended with an appropriate volume of medium.

2.1.13 Phagocytosis of apoptotic Jurkat cells

Jurkat cells were fluorescently labeled with 2 μM CFSE (CellTrace CFSE Cell Proliferation Kit, Molecular Probes) according to the manufacturer's protocol. Apoptosis was induced by UV irradiation (Spectrolink Select XLE-1000 UV crosslinker outfitted with 254nm lamp) of CFSE-labeled Jurkat cells (2 X 10^6 cells/ml in serum-free RPMI) for 15 minutes and cells were further cultured for 4 hr. The percentage of apoptotic cells was quantified by using Annexin-V-FITC Apoptosis Detection Kit II (BD Biosciences) according to the manufacturer's instructions. DCs (1 X 10^5 cells) were incubated with apoptotic cells at a 1:1 ratio in a total volume of 100 μl RPMI 1640 containing 10% human serum for 2 hr at both 37ºC or, as a control, on ice. Phagocytosis was stopped by chilling the cells in an ice slurry. Fluorescence from non-ingested apoptotic cells was quenched with ice cold 0.2% Trypan blue (2 mg/ml in 20 mM sodium acetate and 150 mM NaCl, pH 4.4). Cells were washed 2X in cold FACSwash, fixed with 4% (w/v) PFA, and stained with PE-labelled anti-CD11c prior to flow cytometry analysis.

2.1.14 Determination of cell viability

<u>Annexin-V and propidium iodide staining</u>

The Annexin-V-FITC Apoptosis Detection Kit II (BD Biosciences) was used for this assay. 1 X 10^5 cells were used per staining reaction and resuspended with 100

µl of Annexin-V binding buffer. Then, 5 µl of Annexin-V-FITC and 5µl of propidium iodide (PI) or 7-AAD were added to each reaction and incubated for 15 mins at room temperature in the dark. After incubation, 400 µl of Annexin-V binding buffer was added to each reaction. Cells were analyzed by flow cytometry immediately. Some assays were performed with PI or 7-AAD alone, whereby the cells were resuspended and stained in FACSwash instead of Annexin-V binding buffer.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme that is released upon cell lysis. The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, WI, USA) was used to measure LDH levels in medium to assess cell death according to the manufacturer's protocols. Briefly, cells were cultured at 100 µl/well. Additional wells of the cells were set aside for measuring total LDH released from all cells in the well after complete lysis. 10 µl of lysis solution were added to each of these additional cells and the supernatant was collected 45 min later. Another set of wells were included that contained only culture medium which were used to correct for absorbance contributions caused by phenol red. Cell culture supernatants were collected at the experimental endpoint. 50 µl of supernatant per well was transferred to a microtiter plate. 50 µl of reconstituted Substrate mix was added per well, incubated for 30 min at room temperature in the dark. Finally, 50 µl of stop solution was added per well and the absorbance was measured at 490 nm. The reading at OD450 was performed with the culture medium control and this was used to subtract the readings from the test culture supernatants.

2.2 Molecular Biology Techniques

2.2.1 Total RNA isolation

Total RNA from primary immune cells and cell lines was extracted using the TRIZOL reagent (Gibco BRL Life Technologies, Rockville, MD). Briefly, 1 ml of TRIZOL reagent was added to 0.3 to 1 x 10^6 cells and vortexed vigorously. This mixture is either stored at -80ºC or processed immediately. 0.2 ml of chloroform was added and vigorously mixed. After 2 min, the mixture was centrifuged at 4ºC for 15 min at 12,000X g. The aqueous phase was transferred to a fresh tube and 0.5 ml of isopropyl alcohol was added to precipitate RNA by incubating for 10 min at room temperature. RNA was pelleted by centrifugation for 10 min at 12,000X g, washed with 1 ml 75% (v/v) ethanol and air-dried. The RNA pellet was dissolved in 20 µl of RNAse-free water and stored at -80ºC, or further purified to remove genomic DNA.

RNA obtained after TRIZOL extraction was further purified using Nucleospin RNA II (Clontech, CA, USA) following manufacturer's protocol, and eluted using 40 µl of RNAse-free water. RNA concentration was quantitated using the Nanodrop spectrophotometer and typically the A260/280 ratio is above 1.9. Long-term storage of RNA was performed in -80ºC.

2.2.2 Reverse transcription (RT)

RNA reverse transcription (RT) was performed using the Advantage RT-for-PCR Kit (Clontech Laboratories, Palo Alto, CA). Briefly, RNA (0.2-1.0 µg) was mixed

with 1 µl oligo (dT) primers, heated for 2 min at 70ºC min and rapidly chilled on ice. RT was then performed in a 20 µl reaction volume containing MMLV reverse transcriptase, RNase inhibitor, dNTPs and reaction buffer for 1 hr at 42ºC. The reaction was terminated by heating at 94ºC for 5 min. cDNA was diluted to 100 µl final volume and used immediately or stored at -20ºC.

2.2.3 Quantitative real-time PCR

Real-time PCR reactions were performed in triplicates for each sample in a total reaction volume of 20 µl with the following components -

SYBR Green PCR Master Mix (Applied Biosystems)	10 µl
Forward and reverse primer mix (10 µM each)	1 µl
cDNA	2 µl
Nuclease-free water	7 µl

The reaction mixtures were then run on the ABI Systems 7500 Real-Time PCR machine using the *Comparative Ct quantitation method.* The reaction conditions were 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C. Dissociation curve analysis was always performed at the end of all real-time PCR runs to ensure specificity of primers. The relative expressions of respective genes were normalized to that of GAPDH and calculated usingthe $\Delta\Delta C_t$ method. Table 2.4 provides a complete list of the primers used for real-time PCR detection of gene expression in this study. Most of the primers were designed with the help of PerlPrimer open source software (Marshall, 2004) and whenever possible, the primers would span two consecutive exons such that the amplification of the target gene on unspliced genomic DNA is eliminated.

Table 2.5. Primers used for SYBR Green real-time PCR quantitation of various human genes in this study.

Target human gene	Primers (5'….3') : Forward (Sense) primer / Reverse (Antisense) primer
β-actin	ACCACACCTTCTACAATGA AAACATGATCTGGGTCATCTT
BAFF	ACATTTGTTCCATGGCTTCT TTGCAATGCCAGCTGAATA
C1qA	CTTCCTCATCTTCCCATCT GTTCAGCAGACACAGACA
C1qB	AGGCGTCTGACACAGTATG CCTGGAAGCCCTTTCTCT
C1qC	ACCTGCAGTTCCTTCTCC TTCTCCCTTCTGCCCTTT
CCR7	TTCCAGGTATGCCTGTGT AGGTTGAGCAGGTAGGTA
CD1a	ACGCTGTTTCTGTTAAGAC CCAGGTCAGAACAACAAAT
CD4	CTTCTTAACTAAAGGTCCATCCA AGTCAATCCGAACACTAGCA
CD40	CCAGCCAGGACAGAAAC TACAGTGCCAGCCTTCT
CD40L	CGAAACATACAACCAAACTTCTC TCATCTTCTATCTTGTCCAACCT
CD68	CAGCACAGTGGACATTCTC ATGATGAGAGGCAGCAAGA
cMaf – short isoform	TGAGTCTGACACGCGATT CACCACCACCACAAACTC
cMaf – long	AGCCATCAGTGGGATACG

isoform	AAGGAGACTAAACAGAAGTCAGG
EBI3	TCATAACAGAGCACATCATCAA AGACTCCAGTCACTCAGTT
FoxP3	CTTCATCTGTGGCATCATC TCGCATGTTGTGGAACTT
FZD5	GATCCGTGGAGAGTCCTTTCCCT CAGAGGAATCCGGGCCGG
GAPDH	CGGAGTCAACGGATTTGGTCG TCTCGCTCCTGGAAGATGGTGAT
GATA3	TGCGGGCTCTATCACAAA TCTGTTAATATTGTGAAGCTTGTAGT
GM-CSF	CTGCTGAGATGAATGAAACA AAGTCCTTCAGGTTCTCTTT
IFN-α	CCAGTTCCAGAAGGCTC ATCTCATGATTTCTGCTCTGA
IFN-β	TTGCTCTCCTGTTGTGCT CAAAGTTCATCCTGTCCTTG
IFN-γ	TTGGGTTCTCTTGGCTGTTA TCTGTCACTCTCCTCTTTCC
IFN-γ R1	GCCGAGATGGAAAAATTGGACC TCGCTAACTGGCACTGAATCTCGT
IFN-γ R2	AAACAATGGCAGATGCCTCCACT CGTCATCCTTTGGTGAGCTGTCC
IL-4	CGGGCTTGAATTCCTGTC GCTCGAACACTTTGAATATTTCT
IL-10	AATGCCTTTAATAAGCTCCAAGA TCTCAGTTTCGTATCTTCATTGT
IL-12p35	TTTACCCTTGCACTTCTGA CAACTCCCATTAGTTATGAAAGA
IL-12p40	TGCAGTTAGGTTCTGATCCA CAGCAAAGATATCATTGTGATCCT

IL-17A	CATTGGTGTCACTGCTACTG TGGATCGGTTGTAGTAATCTG
IL-17F	CCTGGAATTACACTGTCACTT ATTCATGGAGATGTCTTCCT
IL-23p19	AATCAGGCTCAAAGCAAGTG TCTTCTCTTAGATCCATGTGTCC
IL-27p28	CTTCCCTTGCTCCTGGTT AGGTGAGATTCCGCAAAG
IRF1	CTCACCAAGAACCAGAGAAA CCCACATGACTTCCTCTT
IRF8	CTGCGTGAATGAAGTTACA TCTGGGAGAATGCTGAAT
M-CSF	CAGGAACAGTTGAAAGATCCA TTGTCAAGGAGATTCTTTGTTTC
NOD1	CACTGTTCTCAGACTCAGCGTAAACC GTTCTTCACAGCCAGGGCGAG
NOD2	GCTAGAAGAACTCTGCCTGGAGGAG GTGTTCCCTCGGAGCCAGACTT
PU.1	AGAGCCATAGCGACCATTA TATCGAGGACGTGCATCT
RORγC	TCATGCCACCTTGAATACAG CCAGGTGCTCTATCTCTGTC
SP1	AAACAGCATATTTGCCACATC CAAATTTCTTCTCACCTGTGT
Stat1	AACAGAAATACACCTACGAACAT CACCAACAGTCTCAACTT
T-bet	AATGTGACCCAGATGATTGT TTGGAAAGTAAAGATATGCGTGTT
TGF-β1	AACCCACAACGAAATCTATGA AATTGTTGCTGTATTTCTGGTA

Wnt5a	GGCCGCAGGACGGTGTACA TGGGCGAGTTGAAGCGGC

Table 2.6. Primers used for SYBR Green real-time PCR quantitation of various mouse genes in this study.

Target mouse gene	Primers (5'….3') : Forward (Sense) primer / Reverse (Antisense) primer
C1qA	ATGACCCTAGTATGGACAGT ATATTGCCTGGATTGCCTTTC
C1qB	AGGTTCTTCCTGCCTCTA GGCTTCCTGTGTATGGAAT
C1qC	AAGGGAGAGCCAGGAATC CTGTGCTTGGGTTGTAATG
CD8α	AAACGAAGGCTACTATTTCTG AGTAGTAGAGTTCACTTTCTGA
CD8β	AAAGATGTCCTGTGAGGTTAA CCACACTTTCACCATACAAA
CD11b	ATCAGTACCAGTTCAACAAC ATCCCATACGGTCACATT
CD11c	TGGAGCTGAATGTAGATGT CTGCTGGTCCTCTTTCTT
CD45	AGAAGAAACCAGCAAATACAT TTTCCAGTAACTCATCACAAAG
GAPDH	CATCTTGGGCTACACTGAG TATTCATTGTCATACCAGGAAAT
Sirp-a	AACCTTCCCTGATAATAATGCT CTTGGCTTTCTTCTGTTTGA

2.3 Protein Chemistry Techniques

2.3.1 Enzyme-linked Immunosorbent Assay (ELISA)

Detection of human cytokines

ELISA was performed using commercially available human cytokine detection kits. The DuoSet® ELISA Development System (R&D Systems Inc, Minneapolis, MN) was used for IL-1β , IL-17 and TNF-α while the BD OptEIA™ ELISA set (BD Biosciences, San Diego, CA) was obtained to quantitate IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70 and IFN-γ and the Ready-SET-Go! Kit (eBioscience) was used for IL-23 detection. Briefly, 100 µl of a capture antibody was diluted with PBS (in DuoSet® ELISA) or Coating buffer (0.1 M sodium carbonate, pH 9.5) (in BD OptEIA and Ready-SET-Go!) was added to each well of a 96-well Maxisorp plates (Nunc, Denmark) and incubated overnight at 4ºC. The plates were washed 3X with PBS-T, blocked with Reagent Diluent (PBS with 1% BSA) (in DuoSet® ELISA) or Assay Diluent (in BD OptEIA and Ready-SET-Go!) for 1 hr at room temperature and washed 3X with PBS-T. Test samples or purified cytokine standards diluted in Reagent Diluent/Assay Diluent were then added into each well (100 µl/well) and incubated overnight at 4ºC. The plates were washed 3X with PBS-T. For DuoSet and Ready-SET-Go! ELISA, the biotinylated detection antibodies diluted in their respective diluents was added to each well (100 µl/well). After incubation at room temperature for 2 hr, the plates were washed and incubated with streptavidin-HRP conjugate in (100 µl/well) for 0.5 hr at room temperature. For BD OptEIA and ELISA, the biotinylated detection antibodies and streptavidin-HRP conjugate were diluted in Assay Diluent, added to each well (100 µl/well) and incubated for 2 hr at room temperature. 100 µl of TMB substrate solution (BD Biosciences) was added

to each well and the plate incubated in dark for 15-30 min. The colorimetrical reaction was stopped by adding 50 µl 1 M sulphuric acid. The absorbance at 450 nm was read immediately with the Model 680 Microplate Reader (Bio-Rad). The linearized standard curve was constructed by plotting the cytokine concentrations versus that of the OD reading value (log scale). The concentration of each sample was derived by comparing with the standard curve and then multiplied by the dilution factors. Results were presented as means of triplicate experiments ± SD.

Detection of secreted C1q by ELISA

For the detection of C1q in cell culture supernatant, an in-house developed sandwich ELISA system was employed. The capture antibody used was polyclonal goat anti-C1q diluted to 0.5 µg/ml in PBS and coated onto Maxisorp plates (100 µl per well) overnight at 4ºC. All washes consist of 3 rounds of washing with PBS-T. The wells were washed and blocked with 3% BSA in PBS for 1 hr. The wells were washed again and samples or purified C1q standards were diluted in 1% BSA in PBS, and added to the wells at 100 µl per well. The plate was incubated overnight at 4ºC. The following day, the wells were washed and a monoclonal mouse anti-C1q antibody (1:2500 dilution in 1% BSA in PBS) was added and incubated for 2 hr in room temperature. The wells were washed and a goat anti-mouse IgG conjugated with HRP (Dako) diluted 1:1000 in 1% BSA in PBS was added. A final wash was performed and TMB substrate was added to quantitate the antibody binding. The reaction was stopped with 50 µl of 1 M sulphuric acid and absorbance was read at 450 nm.

Detection of secreted fibronectin by ELISA

This competitive ELISA assay was adapted from a method described by Pataki *et al* (2006). Maxisorp plate (Nunc) wells were coated overnight at 4ºC with 100 µl of 1 µg/ml purified human fibronectin (in 0.1 M sodium carbonate, pH 9.5). Concurrently, a V-bottom plate was blocked with 200 µl of 1% BSA in TBS for 30 mins. 100 µl of rabbit anti-fibronectin diluted 1:30000 in 0.1% BSA in TBS was then added, followed by 100 µl of purified fibronectin standards or samples diluted in 0.1% BSA in TBS. This plate was also incubated at 4ºC overnight. The Maxisorp plate was then washed 3X with TBS + 0.05% Tween-20. 100 µl of the antigen-antibody mixtures were added to the wells of the Maxisorp plate and incubated for 2 hr at room temperature. The wells were then washed 3X with TBS + 0.05% Tween-20 and 100 µl of anti rabbit-HRP antibody (diluted 1:1000 in 0.1% BSA in TBS) was added and incubated for 2 hr. A final washed was performed with TBS + 0.05% Tween-20 and the TMB substrate was added. The reaction was stopped with 50 µl of 1 M sulphuric acid and absorbance was read at 450 nm.

2.3.2 Antibodies used in this study

The table below gives a detailed account of all the antibodies that were used throughout this study.

Table 2.7. Antibodies used in this study.

Target (human unless stated)	Source	Clone / Isotype (for monoclonal)	Conjugation	Usage	Source
p44/42 MAPK	Rb			W	Cell Signaling
Phospho-p44/42	MS	E10 / IgG1		W	Cell

MAPK (Thr202/Tyr204)					Signaling
p38 MAPK	Rb			W	Cell Signaling
Phospho-p38 MAPK	Rb			W	Cell Signaling
Phospho-Akt (Ser473)	Rb			W	Cell Signaling
Akt	Rb			W	Cell Signaling
SAPK/JNK	Rb			W	Cell Signaling
Phospho-SAPK/JNK (Thr183/Tyr185)	Rb	81E11		W	Cell Signaling
p70/S6K	Rb			W	Cell Signaling
Phospho-p70/S6K (Thr389)	Ms	1A5 / IgG2a			
αV	Ms	MAB1953Z / IgG1		F	Chemicon
β1	Ms	MAB1951Z / IgG1		F	Chemicon
β2	Ms	MAB1962Z / IgG3		F	Chemicon
α2β1	Ms	MAB1998Z / IgG1κ		F	Chemicon
αVβ1	Ms	MAB1999Z / IgG2bκ		F	Chemicon
αVβ3	Ms	MAB1976Z / IgG1		F	Chemicon
αVβ5	Ms	MAB1961Z / IgG1		F	Chemicon
β-actin	Ms	AC15 / IgG1		W	Sigma
Blys /BAFF	Ms	H32-406 / IgG1		ELISA capture	BD
Blys /BAFF	Ms	H32-544 / IgG1	Biotin	ELISA detection	BD
C1q	Ms	IgG1		ELISA, F	Raybiotech
C1q	Go			ELISA, W	Sigma
c-Maf	Rb			W	Santa Cruz
Calreticulin	Rb			F, IF, W	Affinity Bioreagents
CCR7	Ms	150503 / IgG2a	FITC	F	R&D
CD1a	Ms	CB-T6 / IgG1	PE	F	Ancell
CD1c	Ms	AD5-8E7 / IgG2a	APC	F	Miltenyi Biotech
CD3	Ms	UCHT1 / IgG1	None, PE, FITC	A, F	Ancell
CD4	Ms	RPA-T4 / IgG1	Pacific blue	F	BD

CD8	Ms	UCHT4 / IgG2a	PE	F	Ancell
CD11b	Ms	ICRF44 / IgG1	PE-Cy7	F	eBioscience
CD11c	Ms	B-ly6 / IgG1	PE	F	BD
CD14	Ms	UCHM1 / IgG2a	PE, FITC	F	Ancell
CD19	Ms	BU12 / IgG1	PE	F	Ancell
CD21	Ms	HB5 / IgG2a	PE	F	eBioscience
CD25	Ms	BC96 / IgG1	PE	F	eBioscience
CD28	Ms	ANC28.1/5D10 / IgG1		A	Ancell
CD35 (CR1)	Ms	E11 / IgG1		F	Santa Cruz
CD35	Ms	J3D3 / IgG1		F	Santa Cruz
CD36	Ms	FA6-152 / IgG1		F	Beckman-Coulter
CD38	Ms	HB7 / IgG1	PerCP-eFluor-710	F	eBioscience
CD40	Ms	5C3 / IgG1		A, F	eBioscience
CD54	Ms	15.2 / IgG1	FITC	F	Ancell
CD56	Ms	ANC7C7 / IgG1	PE	F	Ancell
CD80	Ms	L307.4 / IgG1	PE	F	BD
CD83	Ms	HB15 / IgG1	PE	F	BD
CD86	Ms	BU63 / IgG1	PE		Ancell
CD91	Ms	A2MR-α2 / IgG1		F	BD
CD93 (C1qRP)	Ms	MAB4314 / IgG2b		F	Chemicon
CD123	Ms	6H6 / IgG1	eFluor-450	F	eBioscience
CD303	Ms	AC144 / IgG1	APC	F	Miltenyi Biotech
Dectin-1	Ms	259931 / IgG2b		B, F	R&D
EEA-1	Rb			IF	Santa Cruz
Fibronectin	Rb			ELISA	Chemicon
FoxP3	Ms	236A/E7 / IgG1	APC	F	eBioscience
gC1q-R/p33	Ms	MAB1160 / IgG1		B, F	Chemicon
gC1q-R/p33	Ms	MAB1161 / IgG1		B, F	Chemicon
IgM	Go	$F(ab')_2$		A	Jackson Immuno
mouse IgG	Go	$F(ab')_2$		A	Jackson Immuno
Interferon gamma	Ms	4s.B3 / IgG1	PE	F	eBioscience
IL-10	Ms	23738 / IgG2b		B	R&D
IL-12	Ms	24910 / IgG1		B	R&D
IL-17A	Ms	eBio64DEC17 / IgG1	Alexa647	F	eBioscience
MHC Class I	Ms	3F10 / IgG2a	FITC	F	Ancell
MHC Class II	Ms	TDR31.1 / IgG1	FITC	F	Ancell

Syntaxin 16	Rb			IF	Synaptic Systems

Keys: Go – goat, Ms – mouse, Rb – rabbit, A – activating, B – blocking, F – flow, IF – immunofluorescence microscopy, W – Western blotting.

2.3.3 Cell lysate preparation

Cells were seeded and stimulated for the specified timepoints before harvesting. The cells were harvested on ice, washed 2X using cold TBS and lysed on ice for 30 min using 100 µl of Biosource (Camarillo, CA) cell lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma) and PhosStop phosphatase inhibitor cocktail (Roche, Basel, Switzerland) according to the manufacturers' recommendations. The lysates are occasionally vortexed to ensure complete lysis. Protein concentration was determined and the lysates are stored at -80ºC until use.

Nuclear extract preparation

Nuclear extracts were prepared from 1 x 10^6 cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, IL, USA) according to manufacturer's specifications. Briefly, cells were harvested in cold by scraping and were washed once in PBS. Supernatant was discarded leaving the cell pellet as dry as possible. Ice-cold CER I (containing protease inhibitors) was then added according to the cell pellet volume as specified by the manufacturer. Cell pellet was resuspended by vigorous vortexing for 15 seconds and then incubated on ice for 10 min. Ice cold CER II was then added and vortexed vigorously for 5 seconds and then incubated on ice for 1 min. Next, cells were vortexed again for 5 seconds before centrifugation maximum speed for 5 min at 4°C. Supernatant containing

cytoplasmic proteins were transferred to a new 1.5 ml pre-chilled tube. The pellet was then resuspended in ice cold NER (containing protease inhibitors), vortexed for 15 seconds and incubated on ice for 40 min. Resuspended pellet was vortexed for 15 seconds after every 10 min during the 40 min incubation. The tube was then centrifuged for 10 minutes at maximum speed at 4°C. Supernatant (containing nuclear proteins) was then transferred to a new 1.5 ml pre-chilled tube. Cytoplasmic and nuclear proteins were stored at -80°C.

2.3.4 Protein concentration determination

Protein concentration was determined using the Bio-Rad protein assay kit. Briefly, eight serial dilutions of a standard BSA solution were prepared, starting with 1.0 mg/ml. 10 µl of the BSA standards and protein samples were added into the wells of a 96-well microtiter plate. The dye reagent was diluted (1 part Dye Reagent Concentrate with 4 parts distilled water) and filtered through 0.45 µm filter. 200 µl of the dye was then added to each well and incubated at room temperature for 5 min. Absorbance was measured at 595 nm. The standard curve was constructed by plotting the known BSA concentrations versus absorbance at 595 nm and sample concentrations determined from the curve.

2.3.5 SDS-PAGE separation of proteins

Typically 5-20 µg of protein samples were run per well and were mixed with 5X Laemmli buffer. The samples were denatured by boiling for 10 min at 95ºC before loading into the wells of a 12.5% SDS-PAGE gel. The gel was run in 1x SDS-

PAGE electrophoresis buffer at 100V until the dye front is approximately at the bottom of the gel.

2.3.6 Western blotting

Proteins resolved by SDS-PAGE were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Immun-Blot® PVDF, Bio-Rad) and incubated overnight at 4°C with blocking buffer (TBS-T containing 5% skim milk). Primary and secondary antibody incubations were done in blocking buffer. The membrane was washed 3 X 5 mins with TBS-T and then visualized using the Immun-Star AP Chemiluminescent substrate (Bio-Rad).

To reprobe a Western blot membrane after chemiluminescent detection, it was first rinsed 3X with TBS-T. Then it was incubated with 10 ml of Western blot stripping buffer in sealed ziplock bags at 50ºC for 20 min with occasional agitation. The membrane was then rinsed 3X with TBS-T and reprobed with another antibody. The efficacy of the stripping process was occasionally tested by incubating the stripped membrane with the chemiluminescent substrate. Absence of signal after 2 hr incubation confirms successful stripping of antibody complexes.

2.3.7 Flow cytometry

Surface staining

Cells were harvested and resuspended at 2 X 10^6/ml in cold culture medium + 1% goat serum. 50 µl of this mix were incubated for 30 min on ice with the respective

antibodies. For cells incubated with fluorochrome-conjugated antibodies, they were washed 2X in FACSwash and fixed with cold 1% (w/v) paraformaldehyde (PFA) in PBS. For non-conjugated antibodies, a second incubation using a PE-conjugated goat-anti mouse antibody was performed. Cells were then washed 2X in FACSwash and fixed with cold 1% PFA. Data acquisition and analysis were performed on Dako Cyan flow cytometer using the Summit 4.3 software (Dako-Cytomation).

Intracellular staining

Cells were harvested and washed 1X with cold culture medium. Fixation and permeabilization was performed using the BD Cytofix/Cytoperm kit. Briefly, ~10^6 cells were fixed with 250 µl of Fixation/Permeabilization solution for 20 min on ice. The cells were then washed 2X in Perm/Wash solution. Fixed and permeabilized cells were then resuspended into 50 µl Perm/Wash solution + 1% goat serum and the respective antibodies were added and incubated for 30 min on ice. Cells were washed 2X in the Perm/Wash solution and resuspended with PBS + 0.1% BSA for analysis. For cells probed with non-conjugated antibodies, they were incubated with a fluorochrome-conjugated secondary antibody against the first antibody on ice for 30 min. The cells were then washed 2X in the Perm/Wash solution and resuspended with PBS + 0.1% BSA for analysis.

2.3.8 Confocal microscopy

DCs were cultured on glass cover slips laid into 24-well plates with the respective stimulation conditions and durations. The cells were washed 3X with PBS and fixed for 20 min in 4% (w/v) paraformaldehyde and washed 3X. The cells were then

incubated for 20 min in 0.1% saponin in PBS at room temperature to permeabilize them, after which primary antibody was then added and incubated for 1 hr. For this incubation, the buffer was supplemented with 1% serum from the species used to develop the secondary antibody. This was followed by three washing steps with 0.1% saponin. Secondary fluorescent-conjugated antibody was then added and the coverslips were incubated in the dark for 1 hr, washed four times with 0.1% saponin, and mounted on slides using VectaShield mounting medium with DAPI (Vector Labs, CA, USA). Confocal images were taken using the Leica TCS SP5 Confocal Microscope System and images were analyzed using the Leica Application Suite.

2.3.9 Live cell microscopy

Cells were examined in live culture using an IX81 inverted microscope equipped with the MicroPublisher 5.0 RTV imaging system (Olympus). Phase contrast images were captured using the Image-pro MC software. 10x objectives were used.

2.4 Experimental repeats and statistical analysis

Most figures presented are results representative of at least 3 independent experiments unless otherwise noted. Data were expressed as mean values of experimental triplicates ± SE (standard error). Analysis for statistical significance was performed using the Student's two-sided unpaired t-test. Where applicable in graphs, * represents $p < 0.05$; and ** represents $p < 0.01$.

2.5 Media and buffers

1 x PBS pH 7.4 (diluted from 10X stock from 1st Base, Singapore)

KH_2PO_4 1.76 mM

Na_2HPO_4 10.4 mM

NaCl 137 mM

KCl 2.7 mM

FACSwash buffer

1 x PBS

Heat-inactivated bovine calf serum 2.5% (v/v)

Sodium azide 0.05% (w/v)

MACS buffer

1 x PBS

0.5% bovine serum albumin

2 mM ETA

Tris buffered saline

Tris-HCL, pH 7.5 50 mM

NaCl 150 mM

10 x SDS-PAGE electrophoresis buffer

Tris base 250 mM

Glycine 2.5 M

SDS 1% (w/v)

5 x Reducing Laemmli buffer

Tris-HCL, pH 6.8 250 mM

Glycerol 50% (v/v)

SDS 10% (w/v)

Bromophenol Blue 0.05% (w/v)

Dithiothreitol (DTT) 0.5 M (added fresh before use)

10 x Western blot transfer buffer

Tris base 250 mM

Glycine 1.92 M

To get a 1X buffer for Western blot, the 10X mix was diluted 1 part to 2 parts 100% methanol and 7 parts deionized water.

PBS-T or TBS-T buffer

1X of PBS or 1X TBS with Tween-20 0.05% (v/v)

Western blot blocking and antibody incubation buffer

Non-fat milk 5%

(prepared in TBS-T buffer)

Western blot stripping buffer

62.5 mM Tris-HCl (pH 6.8)

2% SDS.

0.1M 2-Mecaptoethanol

Chapter 3 Regulation of DC Production of C1q by Various Stimuli

3.1 Introduction

Our previous study (Cao *et al.*, 2003) and that of others (Schwaeble *et al.*, 1995; Castellano *et al.*, 2004) have shown that DCs could produce C1q. In addition, different studies have been performed to examine how the expression of C1q could be regulated by various stimuli, such as LPS (Castellano *et al.*, 2004), steroid drugs like dexamethasone (Armbrust *et al.*, 1997) and cytokines like IFN-γ (Zhou *et al.*, 1991a). However, these studies have utilized different cells type, including macrophages and DCs or cell lines of human, mouse and rat origins. Hence, the effects of these stimuli on these different cell types could be sometimes contradictory. It would thus be advantageous if a unified study on how various stimuli could affect C1q expression on a particular cell type be performed.

In this chapter, we used a human monocyte-derived DC culture system to investigate the regulation of C1q expression by the various stimuli. In our system, the C1q expression could be studied at different levels- mRNA expression was quantitated by real-time PCR, intracellular C1q protein expression was detected by Western blotting and intracellular flow cytometry, and secreted C1q was detected using an in-house sandwich ELISA. We started this project with the ELISA system to screen various stimuli for their effect on C1q production.

3.2 In vitro culture of monocyte-derived dendritic cells (moDC) and its phenotyping

Throughout this study, the majority of experiments were conducted using human DCs differentiated from monocytes isolated from buffy coats of healthy blood donors. The method of Ficoll-gradient centrifugation coupled with plastic adhesion typically yields 1 to 4 X 10^7 monocytes. The purity of these cells was regularly in the range of 90 – 95% as judged by staining with fluorochrome-conjugated anti-CD14 antibody and flow cytometry (Fig. 3.1).

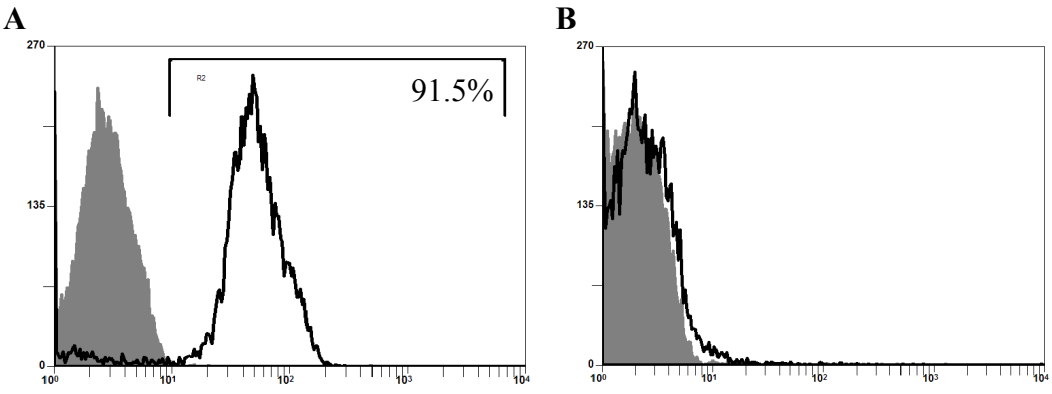

Figure 3.1. Flow cytometry profile of isolated monocytes. PBMCs were isolated from buffy coats using Ficoll-gradient centrifugation and washed repeatedly to remove platelets. Cells were allowed to adhere onto plastic culture flasks for 2 hr. Non-adherent cells were washed away and adherent cells that are mainly $CD14^+$ monocytes were collected by gentle scraping. Isolated cells were then tested by staining with fluorochrome-conjugated antibodies **(A)** CD14 (PE) or **(B)** CD1a (PE). Grey histogram = isotype control, black line = CD14 or CD1a. Flow cytometry analyses were gated on live cells by forward scatter and side scatter.

Human DCs were then differentiated from these monocyte precursors by culturing in the presence of IL-4 and GM-CSF for 6 days, with ½ volume of media being changed and fresh cytokines added every other day. This process was performed in 6-well culture plates where 3 X 10^6 monocytes were seeded per well. After 6 days, the quality of DCs obtained was determined by staining for CD1a positivity and downregulated CD14 expression. Undifferentiated monocytes express a high level of CD14 (Fig. 3.1B) and without surface CD1a (Fig. 3.1A), whereas immature DCs (imDC) obtained after 6 days of differentiation express a high level of CD1a and downregulate CD14 (Fig. 3.2A). We usually obtained about 1.5 X 10^6 DCs per well, about half the number of monocytes originally seeded. Usually about 95% of the cells are CD1a positive, indicating a high purity of the DC preparation.

The functionality of these DCs was confirmed by their ability to up-regulate DC surface markers upon maturation by IFNγ and LPS. ImDC expressed very low levels of CD83 and showed no surface CCR7. These two markers are effectively induced in mature DC (maDC) (Fig. 3.2B). CD40, CD80, CD86, MHC I and MHC II were expressed by imDCs but on mDCs their expressions were further increased. A more homogeneous population of mDC now expressed high levels of these markers as depicted by a more defined peak.

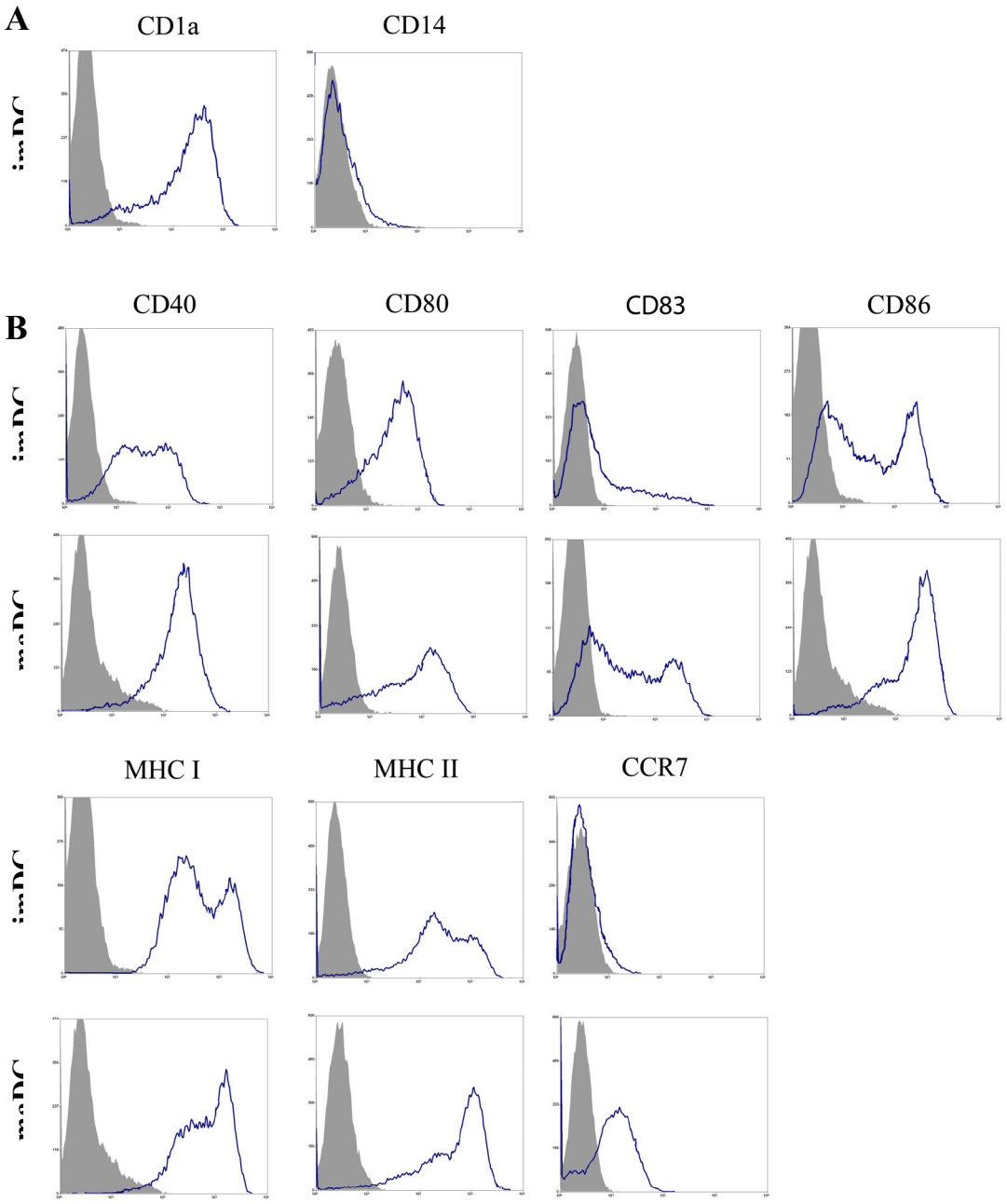

Figure 3.2. Surface phenotype of immature and mature DC. DCs differentiated from monocytes after 6 days of culture were considered as immature DC (imDC). Cells were harvested by gentle scraping, washed 2X with fresh media and stimulated with IFNγ (100 ng/ml) and LPS (500 ng/ml) for 2 days to allow for complete maturation and these are matured DC (maDC). ImDC were stained for CD1a and CD14 (A). CD40, CD80, CD83, CD86, MHC I, MHC II and CCR7 antibodies were used to stain both imDC and mDC (B). Filled histograms are signals obtained with isotype IgG; Open histograms are staining with specific antibodies.

3.3 Establishing a system to detect DC expression of C1q in the levels of transcription, translation and secretion

Upon acquiring DC phenotypes after 6 days of differentiation, we studied C1q expression in the cells. We harvested DCs after 6 days of differentiation, extracted total RNA from the cells, and converted the mRNA to cDNA. As a comparison, we also studied the mRNA obtained from undifferentiated monocytes and macrophages. We used the cDNA to perform a quantitative real-time PCR assay to detect the expression of all 3 C1q chains, C1qA, C1qB and C1qC. In this assay, cDNA was amplified by PCR, and the SYBR Green dye binds the amplified double-stranded DNA. As more copies of dsDNA were amplified, more SYBR Green would be bound and it results in increased fluorescence which was detected by the machine.

DCs expressed significantly higher levels of all 3 C1q chains compared to monocytes (Fig. 3.3). In looking at the relative expression levels of the mRNA, whereby the expression level of each of the 3 C1q chains in monocytes is set to 1, C1qA expression on DC for C1qA, B and C respectively was 20-fold, 40-fold and 140 fold higher. By comparison, macrophages differentiated under the presence of M-CSF have the highest level of C1q expression, about 400-fold higher for both C1qA and C1qB mRNA, and about 1800-fold higher for C1qC. Thus at the transcription level, DCs expressed all 3 C1q chains.

Some of the DCs obtained were washed with PBS and lysed with a cell lysis buffer and subjected to SDS-PAGE. The proteins on the gel were then transferred to a PVDF membrane, which was then probed with a polyclonal goat anti-C1q antibody. The inclusion of DTT in the sample loading buffer prior to SDS-PAGE

electrophoresis will dissociate the C1q macromolecule into its 3 constituent A, B and C chains. This is because the A-B and C-C chains are held together by disulphide bonds which are broken under the reducing conditions in the presence of DTT. From our experience, the goat anti-C1q antibody gives a much stronger signal with the C1qA chain than with the C1qB and C1qC chains.

In Fig. 3.4A, monocyte lysates did not give any signal with the C1q antibody, while the lysates of macrophages had high levels of C1q. C1q was also detected in DC lysates but at a lower level than in macrophages. This was further confirmed by intracellular staining of C1q, where macrophages expressed more C1q than DCs (Fig. 3.4B). The advantage of intracellular C1q staining is that the mouse monoclonal antibody used detects the globular domain of C1q, which is a part of the overall C1q macromolecule structure. Thus, it is specific in detecting the completely assembled C1q whereas there is a chance that Western blotting could also detect unassembled C1q chains.

The final assay performed to confirm C1q expression in DC was a sandwich ELISA to quantitate the levels of secreted C1q in culture supernatant. A goat polyclonal anti-human C1q antibody was coated onto an ELISA plate to be used as the capture antibody. The ELISA plate was then blocked with BSA and diluted samples together with separately prepared C1q standards were then loaded. C1q in the samples or standards were bound by the capture antibody, following which the mouse monoclonal antibody against the globular domain of human C1q was used as a detection antibody. Finally, a HRP-conjugated goat anti-mouse antibody was used to detect the binding of the detection antibody to C1q. The dual layer of specificity

conferred by the two antibodies against human C1q allows for detection of C1q in RPMI 1640 medium supplemented with bovine serum. We have confirmed that the ELISA does not detect bovine C1q from the bovine calf serum (Fig. 3.5).

Differentiated DCs and macrophages were harvested, washed and reseeded into 96-well plates. The cells were incubated for two days before the supernatant was harvested and tested for C1q. As shown in Fig. 3.5, DCs secreted C1q typically in the range of 0.5 to 5 X 10^1 ng/ml levels. In agreement with the C1q levels as detected on the mRNA and cell lysate, macrophage secreted more C1q than DC, in this case about 85 ng/ml compared to 40 ng/ml by DC.

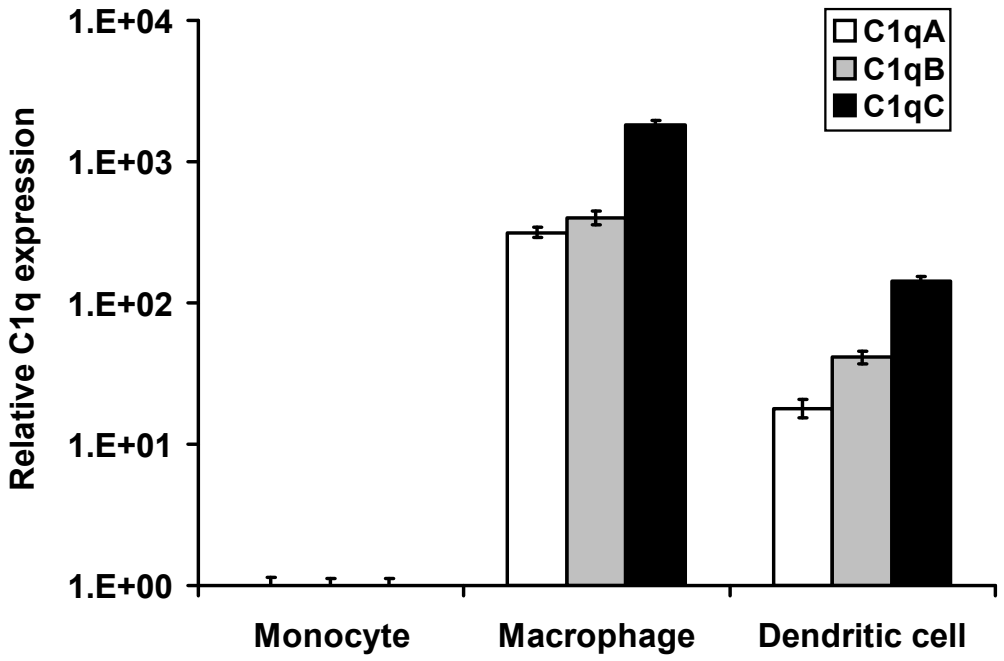

Figure 3.3. Real-time PCR quantitation of mRNA from monocyte, macrophage and DC for C1q expression. Total mRNA from the 3 cells were extracted, then reverse transcription performed to obtain cDNA and the levels of C1qA, C1qB and C1qC mRNA expression was quantitated using real-time PCR and normalized to GAPDH expression. The expression levels for macrophage and DC represents the fold increase over that of monocytes.

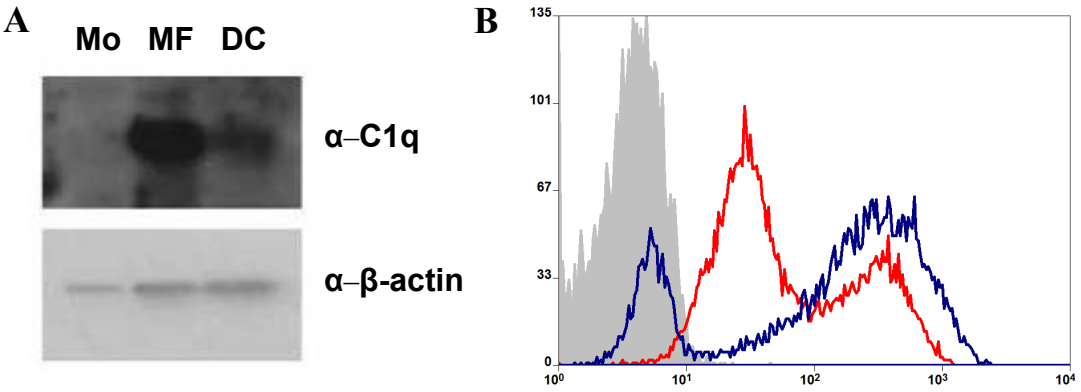

Figure 3.4. Intracellular C1q detection in monocytes, macrophages and DCs via Western blot and flow cytometry. (A) About 20 µg of total cell lysates were resolved via SDS-PAGE and electroblotted onto a PVDF membrane. A goat polyclonal anti-C1q antibody was then used to probe the membrane. The membrane was stripped and re-probed with an anti-β-actin antibody. Mo – monocyte; MF – macrophage; DC – dendritic cell **(B)** DC and MF cultured for 2 days were harvested, fixed and permeabilized. A mouse anti-C1q antibody or isotype control was used to stain intracellular C1q and a PE-conjugated goat-anti mouse IgG was used to visualize the C1q staining. Grey histogram – isotype control; Red histogram – DC, Blue histogram – Macrophage.

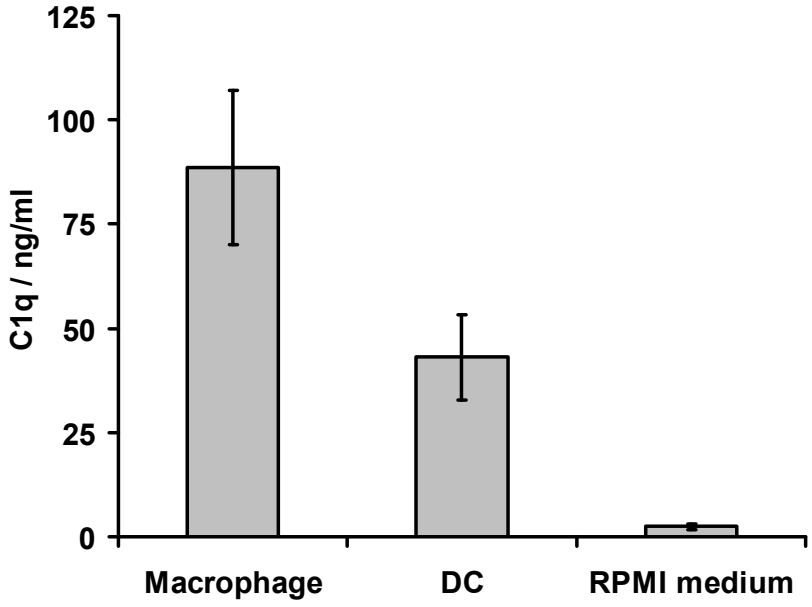

Figure 3.5. Quantitation of secreted C1q in cell supernatant. Macrophage and DC were cultured at a density of 7 X 10^4 cells per well (in 200 µl per well of RPMI 1640 medium supplemented with 10% heat-inactivated BCS in 96-well U-bottom plates) for 2 days. Supernatant was collected and the secreted C1q was quantitated using a sandwich capture ELISA assay with purified C1q as standards. RPMI medium without any cells was used as a blank control. Experiments were performed in triplicates and the results are presented as means ± SD.

3.4 Regulation of C1q production in DC

Having established suitable assays to quantitate C1q production in moDCs, we then proceeded to utilize the ELISA method to screen a large amount of different stimuli for their ability to modulate C1q production in these DCs. We seeded DCs in 96-well round bottom plates and stimulated them for 2 days, collected the culture supernatant, washed the cells and cultured them for another 2 days in fresh culture medium without adding back the stimuli. Thus for each stimulus, there are two sets of results, one for the first 2 days and another for the subsequent 2 days (2 + 2 days).

The large number of stimuli used required that many separate experiments be performed to complete the screening. The results from each experiment were then tabulated, the effect on C1q production by each stimulus was expressed as a fold-change over the unstimulated DC, and then the fold-change from multiple experiments were averaged and presented in Fig. 3.6 and Fig. 3.7. As the results presented in these two figures were averaged from a few independent experiments, the standard deviations of the fold-changes appear large in mose cases.

In Fig. 3.6, the effects of various microbial stimuli on C1q production are shown whereas in Fig. 3.7, the effects of steroid drugs, hormones and cytokine/chemokines are presented. Typically, we observed that unstimulated DCs produce 2 to 3 times more C1q in the 2 + 2 days supernatant compared to the 2 days samples (Fig. 3.12 and Fig 3.13). Table 2.1 summarizes all the microbial stimuli used and the pattern recognition receptors that they engage. The TLR3 and TLR5 ligands Poly(I:C) and flagellin had minimal effect on C1q production and so was the TLR7/8 ligand CL097. LPS, which stimulates TLR4 signaling, upregulated C1q strongly in DCs,

although in 2 experiments we observed that LPS had no effect. LTA which stimulates TLR2, and PGN which is a particulate ligand which stimulates both TLR2 and NOD1/2 also did not affect C1q production after 2 days of DC stimulation. However, both LTA and PGN increased C1q production after DCs were washed and cultured for another 2 days. The more specific NOD1 ligand TriDAP and NOD2 ligand MDP had minimal effect on C1q production. The synthetic agonists Pam3CSK4, Pam2CSK4 and FSL-1 which stimulate TLR2 in dimers with TLR1 or TLR6 all increased C1q production in both 2 and 2 + 2 days culture. Three different forms of CpG DNA were used in this experiment and all downregulated C1q production in DCs for both time-points. Interestingly, zymosan which also stimulates TLR2 together with TLR4 and TLR6 reduced C1q production.

We also studied the effects of steroid drugs on C1q production in DCs. Cortisone, dexamethasone, prednisone and hydrocortisone all slightly reduced C1q secretion with 2 days stimulation but had minimal effects for 2 + 2 days stimulations. The female hormone estradiol showed the same effects but 3 other hormones progesterone, insulin and HCG reduced C1q secretion by about one third for the 2 time-points. The appetite-inhibiting hormone leptin had no effect on C1q secretion.

Among the chemokines and cytokines tested, the chemotactic peptide fMLP, IL-1β, IL-6, M-CSF, soluble CD40 ligand, TGF-β and a combination of GM-CSF with IL-4 had minimal effect on DC secretion of C1q. Osteoprotegerin, thymic stromal lymphopoietin and TNF-α inhibited C1q secretion roughly by half for both time-points. We noticed that GM-CSF on its own reduced C1q levels by about one-third for 2 days but after 2 + 2 days, it had no effect. In contrast, IL-10 also reduced C1q

levels by the same margin for 2 days, but C1q levels were double that of unstimulated cells in the 2 + 2 days sample.

Interestingly, the type 2 interferon IFN-γ strikingly increased C1q production for both 2 days and 2 + 2 days samples. In contrast, the type 1 interferon IFN-α had no effect on C1q level with 2 days of stimulation, but it decreased C1q secretion in the long term 2 + 2 days culture.

3.5 Expression of C1q in primary human plasmacytoid DC and CD1c$^+$ myeloid DC from peripheral blood leukocytes

We then sought to examine the expression of C1q in DCs isolated from the blood of healthy donors. We chose two frequently studied DCs from this group, plasmacytoid DC (pDC) and the CD1c$^+$ myeloid DC (mDC) for this study. Firstly, PBMCs were purified from buffy coat of healthy donors as in the monocyte isolation procedure. Then, pDC and mDC were isolated from the total PBMCs using the relevant antibody-coated magnetic bead MACS kits from Miltenyi Biotec. Due to the rarity of these two cells, at least 1 X 10^8 PBMCs were used mDC isolation and about 2 X 10^8 PBMCs were used for pDC isolation. This resulted in a yield of approximately 0.5 X 10^6 for each cell type.

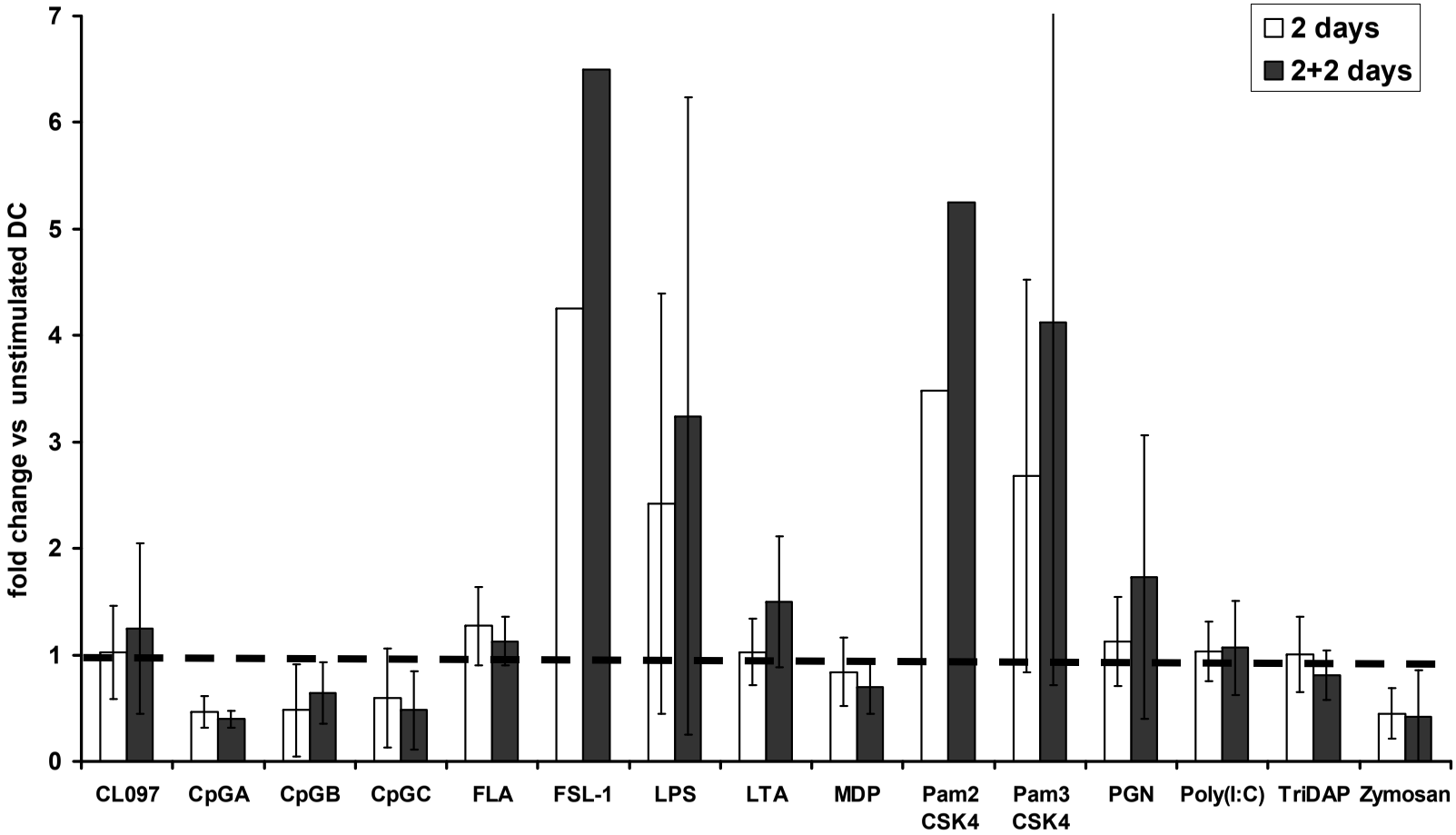

Figure 3.6. Differential regulation of C1q production in DCs by various microbial stimuli. A panel of inflammatory ligands was used to stimulate PRRs on DCs seeded in a U-bottom 96-well plate. The results shown here represent the mean fold change in C1q expression compared to unstimulated cells from different experiments. The white bars represent culture supernatant collected after 2 days of maturation and the grey bars are for cells that were washed after 2 days and incubated for 2 more days.

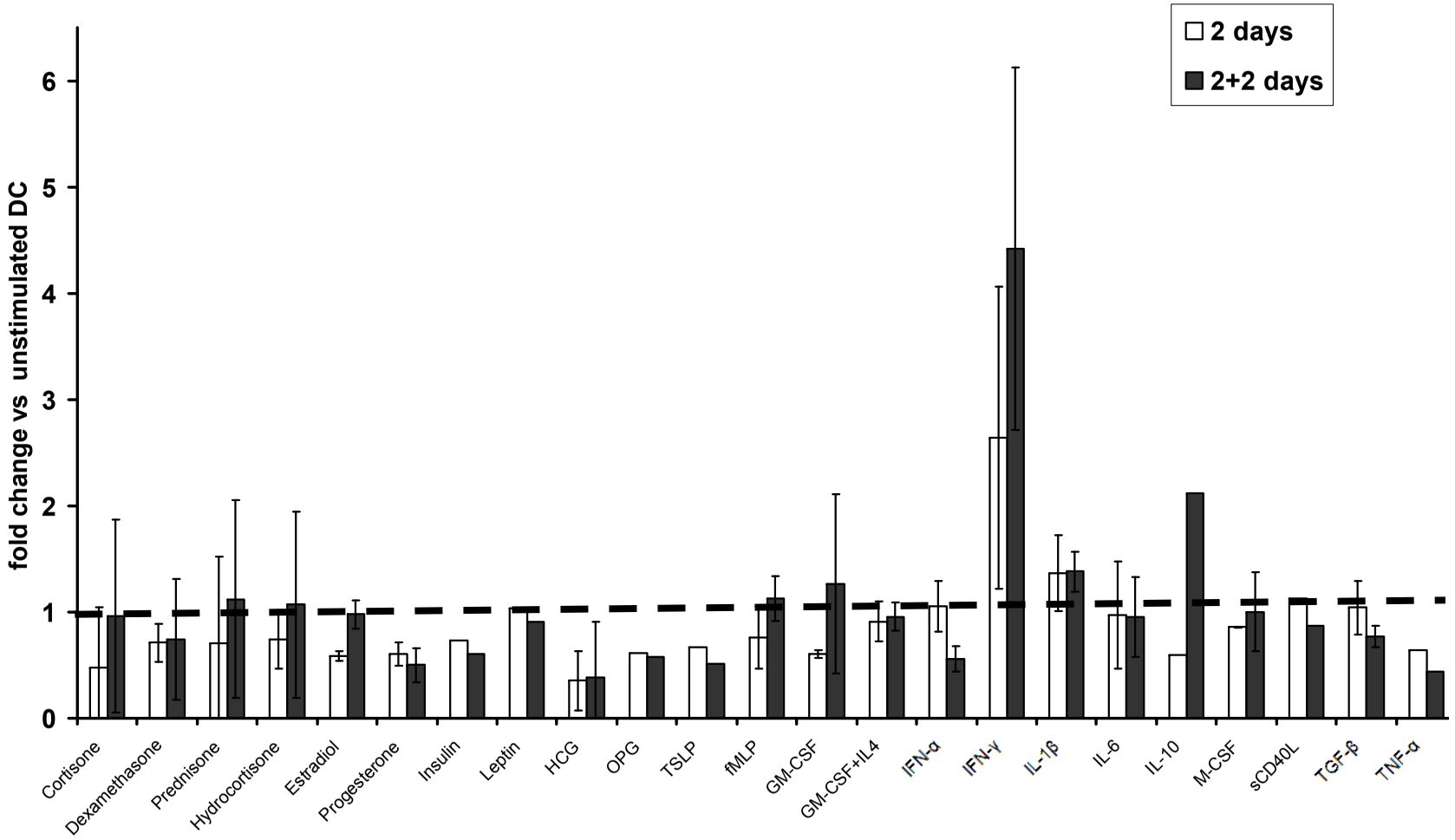

Figure 3.7. Differential regulation of C1q production in DCs by steroid drugs, hormones and cytokine/chemokines. A collection of stimulants was used to stimulate DCs seeded in U-bottom 96-well plates. The results here represent mean fold change in C1q expression compared to unstimulated cells from different experiments. The white bars represent culture supernatant collected after 2 days of stimulation and the grey bars are from cells that were washed after 2 days and cultured for 2 more days.

For the isolation of pDCs, a cocktail of biotin-conjugated antibodies targeting T cells, B cells, NK cells, mDC, monocytes, granulocytes and erythroid cells was added and then subsequently anti-biotin magnetic beads were added. The non-pDC cells were then magnetically depleted, and pDCs were directly labeled with anti CD304 (BDCA-4/Neuropilin-1) magnetic beads and positively selected. The purity of isolated pDC was confirmed by double staining with CD123 and BDCA-3/CD303. The scatter profile of the total PBMCs prior to isolation showed heterogeneity in the cell population whereas the isolated pDC was a highly homogeneous population (Fig. 3.8A). Staining for CD123 and BDCA-3 in total PBMCs showed about 1% of the cells expressing both markers whereas more than 97% of the purified pDCs were CD123 and BDCA-3 double positive, indicating that the enriched cells were highly pure (Fig. 3.8B).

The MACS method was also used to isolate mDCs. In this purification, PBMCs were labeled with anti-CD1c-PE and anti CD20 magnetic beads targeting B cells. The first round of magnetic purification depletes B cells, followed by labeling of the $CD1c^+$ cells with anti-PE magnetic beads. The mDCs were then directly separated via a magnetic column. Purified mDCs were tested for CD1c expression and the absence of B cells via CD19 staining, as some B cells express low levels of CD1c. In Fig. 3.9C, more than 91% of the purified mDC cells were $CD1c^+$ and about 4% of cells in this fraction expressed CD19 (Fig. 3.9B). Thus B cell contamination was at a minimum. In comparison, less than 5% of total PBMCs stained positive for CD1c (Fig. 3.9C) and the B cell fraction represented 2.4% of the total cells (Fig. 3.9B).

Total RNA was isolated from freshly purified mDC and pDC together with moDC differentiated after 6 days of culture, and all RNA were converted to cDNA for real-time PCR analysis. Quantitation of C1q A, B and C chain mRNA across all 3 cell types revealed that mDC and pDC expressed about a 100-fold less C1q than moDC for each of the 3 chains (Fig. 3.10). Thus mRNA quantitation alone suggests that mDC and pDC expressed much lower levels of C1q than moDC.

Next, we seeded the isolated mDC, pDC and cultured moDC in U-bottom 96-well culture plates for 2 days and collected the supernatant. The cells were then washed once and cultured for another 2 days with fresh medium. Supernatant from these 2 + 2 days cultures were also collected and analyzed by ELISA for C1q levels. moDC produced 4.5 ng/ml of C1q after 2 days culture while mDC secreted 1.5 ng/ml (Fig. 3.11). When cultured for 2 + 2 days, moDC expressed 21 ng/ml of C1q while mDC expressed 3 ng/ml. Addition of IFN-γ to the culture increased C1q secretion by moDC to 15 ng/ml and 45 ng/ml for the 2 days and 2 + 2 days culture respectively, while mDC treated with IFN-γ also increased C1q expression to 5.5 ng/ml for 2 days culture and 14 ng/ml for 2 + 2 days culture. In all cases, the secretion of C1q by pDC was below the detection levels of the ELISA.

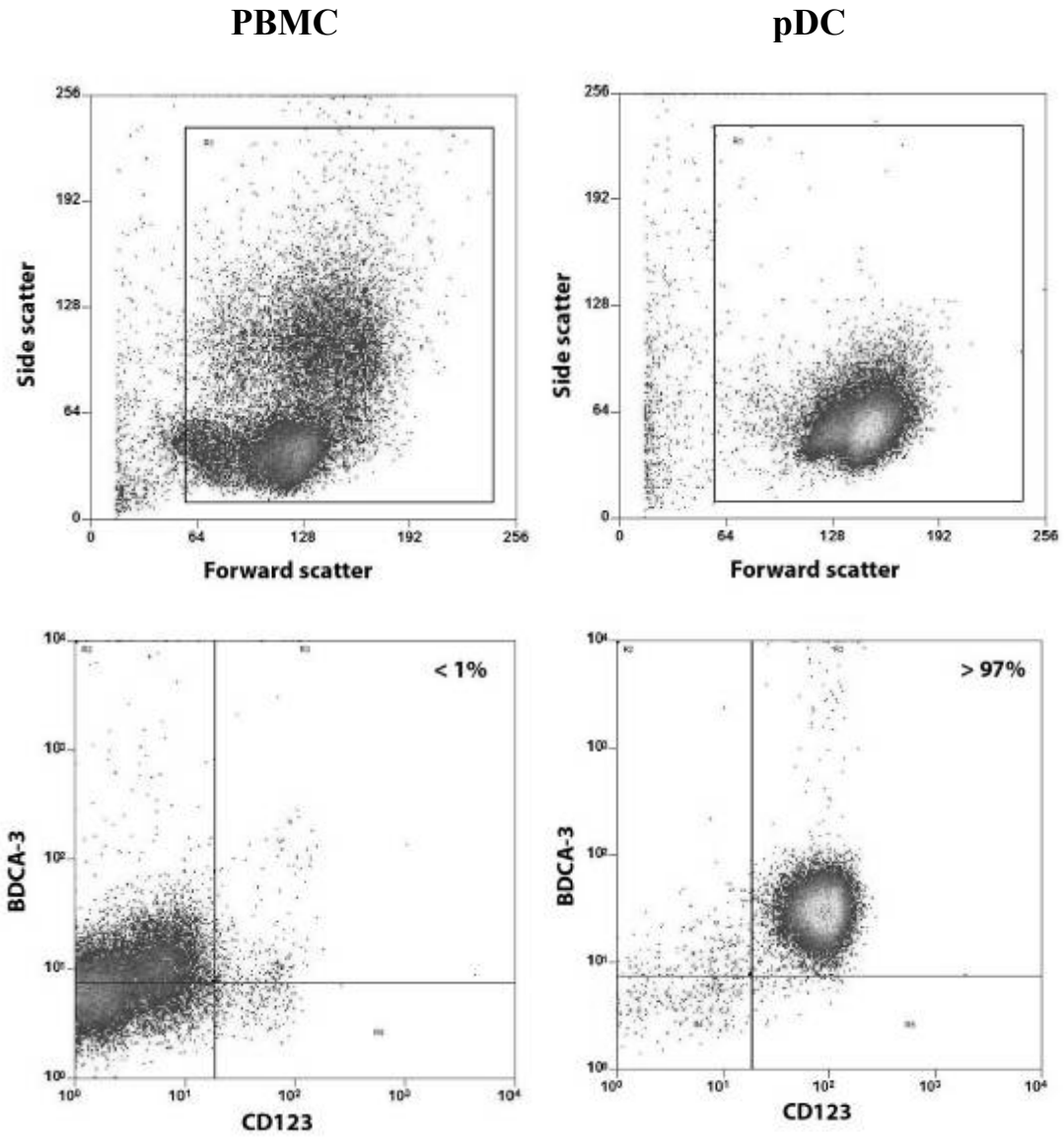

Figure 3.8. Flow cytometry profile of total PBMC and isolated pDC. Total PBMC and magnetically isolated pDC were stained with anti-CD123 and anti-BDCA-3 and analyzed via flow cytometry. The forward and side scatter profiles are shown in the upper panel. Live cells were then gated according to the large rectangle gate in the scatter profiles and the cells were analyzed for CD123 and BDCA-3 staining (lower panel). The values shown in the lower panel represent the percentage of cells double staining for CD123 and BDCA-3.

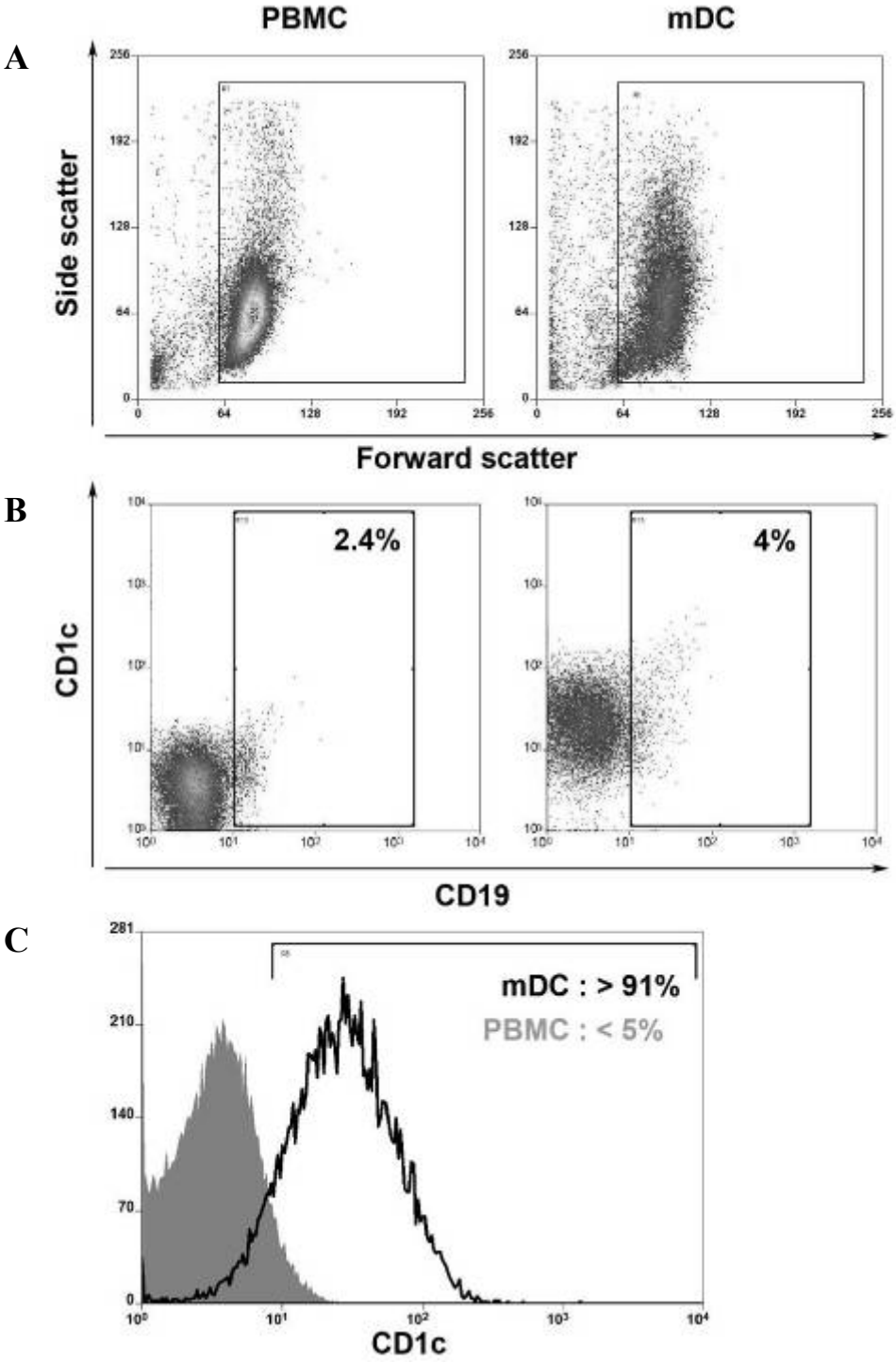

Figure 3.9. Flow cytometry analysis of PBMC and purified mDC. Total PBMC and mDC isolated via MACS were stained with anti-CD1c and anti CD19 and analyzed via flow cytometry. **(A)** The forward and side scatter profiles of both PBMC and mDC are shown. **(B)** Live cells were then gated according to the large rectangle gate in the scatter profiles for subsequent analysis. The percentage of $CD19^{+}$ B cells is given in the boxed region. **(C)** The black hollow histogram represents CD1c expression by mDC and the grey shaded histogram shows CD1c expression in PBMCs. The values shown are the percentage of cells positively staining for CD1c.

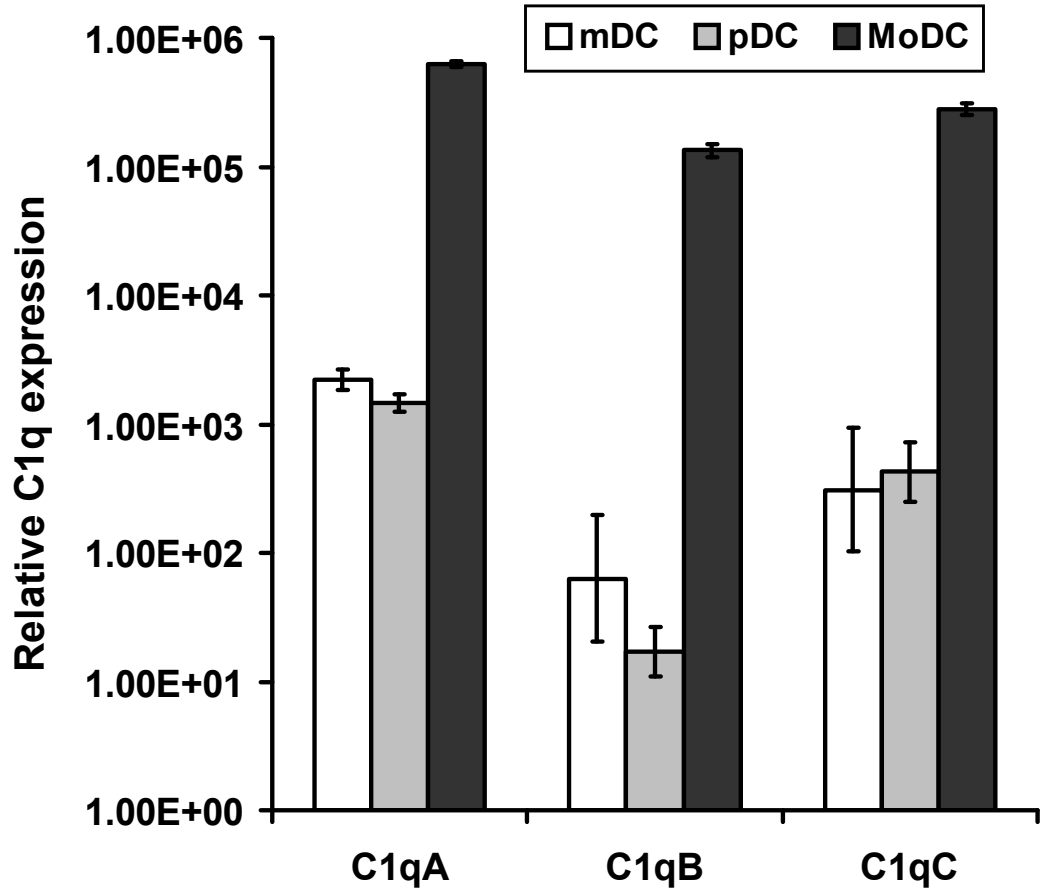

Figure 3.10. Quantitation of the expression of C1q A, B and C chains mRNA in mDC, pDC and moDC. Total mRNA was extracted from about 0.3 X 10^5 cells of freshly isolated mDC and pDC and immature moDC obtained after 6 days of differentiation. 0.3 μg of total RNA was converted to cDNA and then analyzed using real-time PCR. The values on the Y-axis are the expression levels of the genes relative to each cell type.

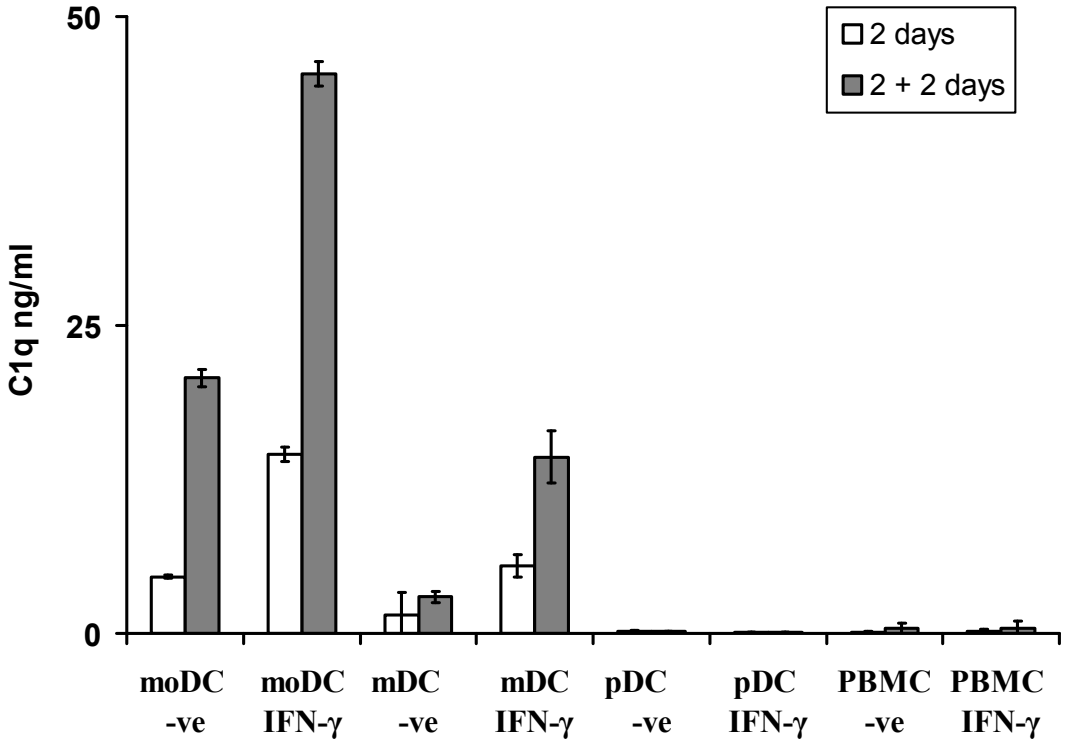

Figure 3.11. ELISA detection of C1q secreted by moDC, mDC and pDC into culture supernatant. 5 X 10^4 moDC, mDC and pDC together with total PBMC were cultured in triplicates with or without IFN-γ (100 ng/ml) in U-bottom 96-well plates. Supernatant was collected after 2 days, then the cells were washed 1X with fresh medium and cultured for another 2 days. The supernatant was collected again (2 + 2 days). Secreted C1q in both sets of supernatant was quantitated using a sandwich capture ELISA assay with purified C1q as standards. Experiments were performed in triplicates and all results are presented as means ± SD.

3.6 Expression of C1q in mouse BMDC and splenic DCs

We then expanded from studying C1q production in human DCs to mouse DCs. For this study, we performed real-time PCR assay using specific primers for mouse C1qA, B and C genes. Total RNA from bone marrow-derived DC (BMDC) that was obtained from Dr Wong Siew Heng (Department of Microbiology, National University of Singapore) was extracted and converted to cDNA. Three functionally distinct DC subsets can be defined in a steady state mouse spleen. These are the plasmacytoid DC (pDC), $CD8^+$ conventional DC (cDC) and $CD8^-$ cDC. They were enriched and FACS sorted from pooled mouse spleen in the laboratory of Professor Ken Shortman, The Walter and Eliza Hall Institute, Melbourne. The cells were shipped to our lab as frozen samples and we extracted RNA using our standard protocol, and converted the RNA to cDNA. As a control, we also used cDNA from the mouse RAW264.7 cell line, which does not produce C1q in our tests.

Mouse DC subtypes sorted at the laboratory of Professor Ken Shortman were routinely >98% pure (Vremec *et al.*, 2000; Lahoud *et al.*, 2006). We verified the DC purity by performing real-time PCR analysis on the cDNA for genes that are known to be markers for these different DC subtypes – CD8α, CD11b, CD11c and SIRPα. All DCs expressed high levels of CD11c, with pDC showing the lowest expression (Fig. 3.12). BMDC and $CD8^-$ cDC expressed high levels of CD11b, with pDC showing intermediate levels and $CD8^+$ cDC the lowest. CD8α expression was highest in $CD8^+$ cDC while pDC also showed relatively high expression of this gene. $CD8^+$ cDC expressed the lowest levels of SIRPα compared to the other 3 DCs tested, and SIRPα expression was high in $CD8^-$ cDC that are known to express this marker. The relative expression of these markers in our sorted mouse DCs is

consistent with their reported phenotypes (Lahoud *et al.*, 2006; Shortman and Heath, 2010) and should thus be of high purity.

For the analysis of C1q mRNA, C1q expression in RAW264.7 cells was set to 1 as the calibrator for relative quantitation. BMDCs produced the highest level of C1q mRNA, 7 X 10^5 fold higher than RAW264.7 for C1qA, 4 X 10^4 fold more for C1qB and for C1qC, 3 X 10^4 fold higher (Fig. 3.13). This was then followed by $CD8^-$ cDC which produced half the amount of C1qA as BMDC and about 10-fold less C1qB and C1qC. Next, pDC produced 15-fold less C1qA and 10-fold less C1qB and C1qC than $CD8^-$ cDC. The $CD8^+$ cDC expressed the lowest levels of C1q of all the mouse cells tested, 8-fold less C1qA and C1qC and 10-fold less C1qB than the $CD8^-$ cDC.

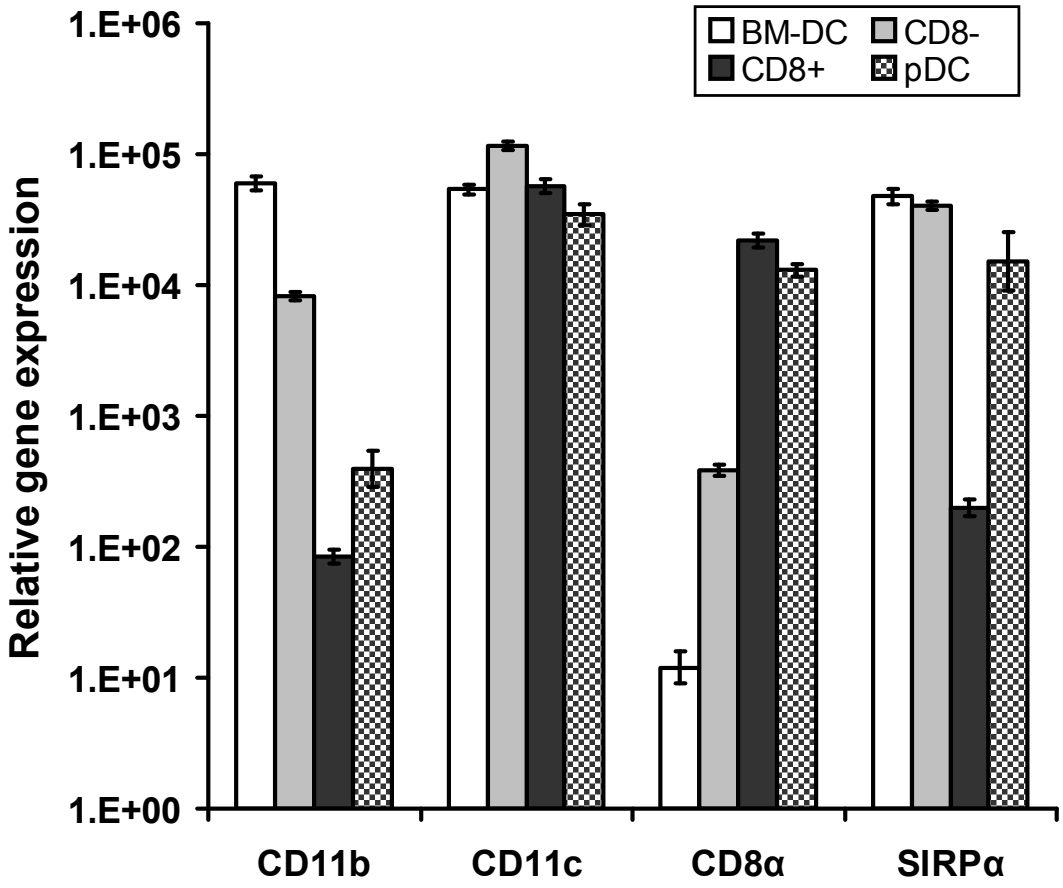

Figure 3.12. mRNA expression of various markers for subtyping mouse DCs. BMDCs were differentiated from mouse bone marrow cells for 7 days with GM-CSF and total RNA was extracted from these cells. Splenic $CD8^+$, $CD8^-$ and pDC cells were obtained from the lab of Professor Ken Shortman in the Walter and Eliza Hall Institute, Melbourne, in frozen form. Total RNA was then extracted. As a control, RNA was also extracted from the mouse macrophage cell line Raw264.7. All RNA were converted to cDNA and subjected to quantitative real-time PCR analysis using mouse CD11b, CD11c, CD8α, SIRPα and β-actin specific primers. The bars show the relative gene expression levels normalized to β-actin.

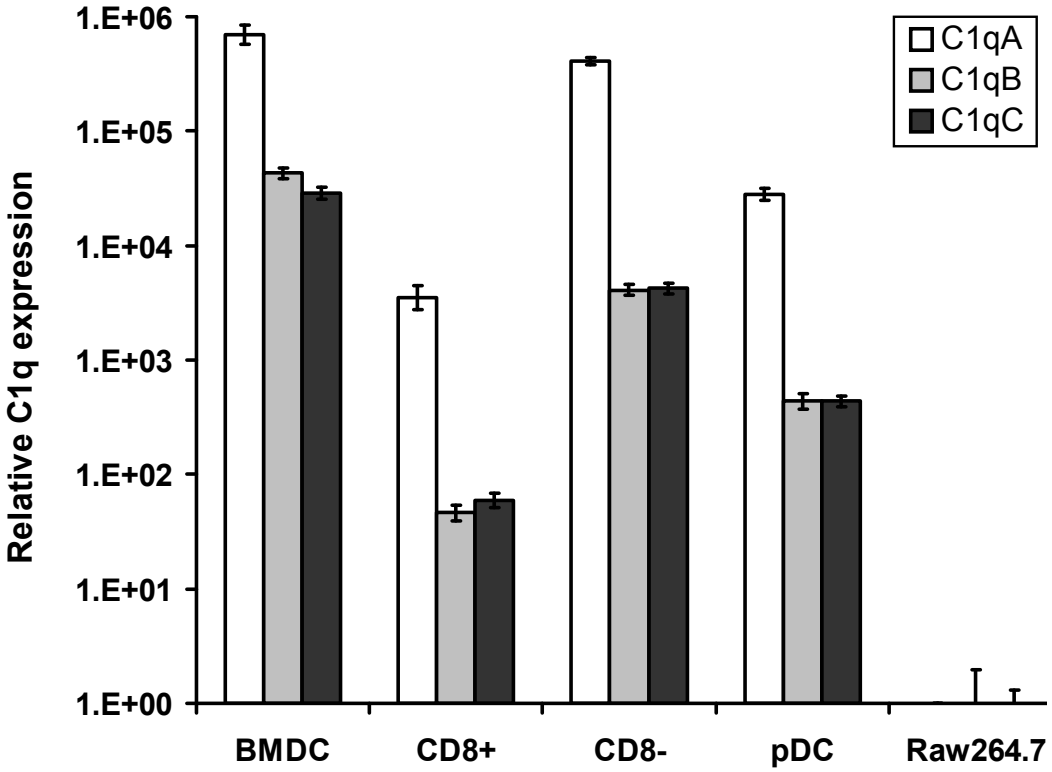

Figure 3.13. Mouse DCs express C1q mRNA. The same cDNA samples in Fig. 3.14 were used in this test where quantitative real-time PCR analysis using mouse C1qA, B and C and GAPDH specific primers was performed. The bars show the relative expression of mouse C1q genes and the results are normalized to β-actin. C1q expression in Raw264.7 cells was set as the calibrator (value = 1).

Chapter 4 Suppression of C1q production in DC by the yeast-derived stimulus zymosan through Dectin-1

4.1 Introduction

In a large scale screening study in Chapter 3.4, we examined a number of factors that are potentially related to SLE pathogenesis or factors which have been studied in previous studies on C1q regulation for their ability to modulate C1q production in moDC. An ELISA system has been developed for C1q detection and it provides sensitive detection of C1q in cell culture medium. Our results showed that a yeast-derived structure, i.e. zymosan, can significantly inhibit C1q production in DC. Zymosan is a ligand for TLR2, but curiously other TLR2 ligands did not downregulate C1q production. Recently it has been highlighted that zymosan also stimulates a C-type lectin receptor called Dectin-1/Clec7A (Reid *et al.*, 2009).

We then performed a series of experiments to dissect the underlying mechanisms by which zymosan inhibits C1q production. We found that Dectin-1 indeed appears to be engaged by zymosan and this leads to the observed inhibition of C1q production. We then sought to study the downstream signaling events transduced by Dectin-1 ligation that led to the inhibition of DC production of C1q.

4.2 Zymosan down-regulates C1q production in DC

We utilized the assays for analyzing C1q production that were developed in Chapter 3 to study how zymosan suppresses C1q production in DCs. First, we sought to determine the optimal concentration of zymosan to use. We stimulated DCs with zymosan at different concentrations for 2 days and 2 + 2 days and analyzed the culture supernatant for C1q via ELISA. As shown in Fig. 4.1, zymosan downregulates C1q production by DCs is a dose-dependent manner. The inhibitory effect of zymosan was more obvious after prolonged stimulation. In the first 2 days, zymosan only showed significant inhibition on C1q secretion by DCs when it was used at 50 μg/ml or higher. At 10 μg/ml, it had no detectable effects. However, cells stimulated at 10 μg/ml in the first 2 days showed markedly reduced C1q production in the next 2 days. From this set of titrations, further experiments were conducted using zymosan at 50 μg/ml in order to observe its effects in the first 2 days.

Next, we proceeded to study if the inhibition of C1q production occurs at the transcription and translation levels. We lysed DCs after 2 days of culture without any stimuli or with zymosan. The lysates were subjected to Western blotting and probed for C1q. After 48 hrs of stimulation with zymosan, intracellular C1q levels in DCs were drastically reduced (Fig. 4.2) compared to unstimulated cells.

We progressed to look at the transcription of C1q mRNA in DCs stimulated with zymosan. We stimulated the cells for 5 hr, 1 day and 2 days and extracted total RNA, converted it to cDNA and analyzed via real-time PCR. For 5 hr and 1 day, no obvious changes were observed in C1qA and C1qB mRNA expression but C1qC mRNA expression was lower after zymosan treatment (Fig. 4.3). After 2 days of

zymosan stimulation, mRNA expression of all C1q chains was consistently reduced. For C1qA, it was reduced by 1.5-fold, for C1qB, the reduction was 2-fold and it was a 4-fold reductin for C1qC.

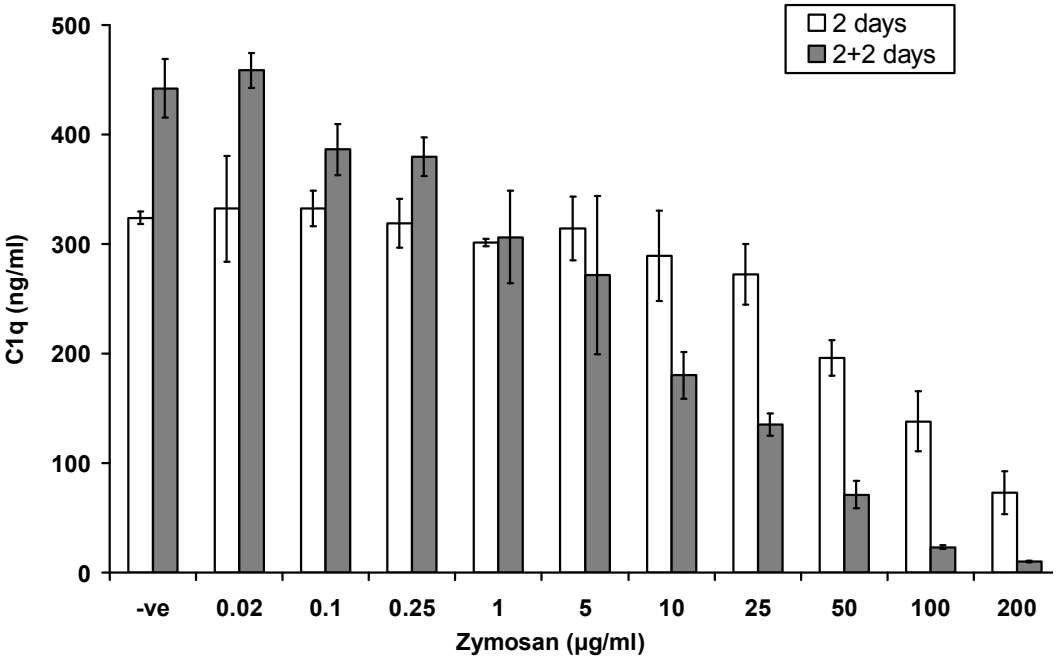

Figure 4.1. Dose dependent suppression of C1q secretion by DC following zymosan treatment. 7 X 10^4 DCs were seeded in 96-well U-bottom plates and treated with increasing concentrations of zymosan. Supernatant was collected after 2 days, cells washed and cultured for 2 more days (2 + 2 days). C1q levels in culture supernatant were measured by ELISA. This experiment was performed twice. Stimulations were performed in triplicates and results are presented as means ± SD.

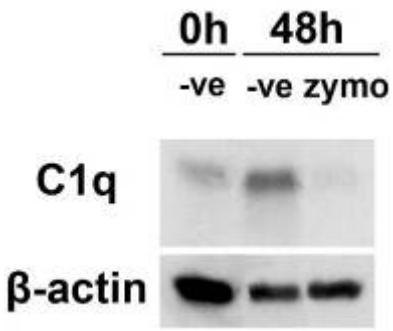

Figure 4.2. Western blot of total DC lysate for C1q and β-actin after zymosan stimulation. 10^6 cells were either unstimulated when harvested on day 6 of DC differentiation (0h -ve), or cultured for a further 48 hrs with zymosan (48h zymo) or without stimuli (48h -ve) and then lysed and analysed via Western blotting with an antibody specific for C1q. The membrane was then stripped and re-probed for β-actin.

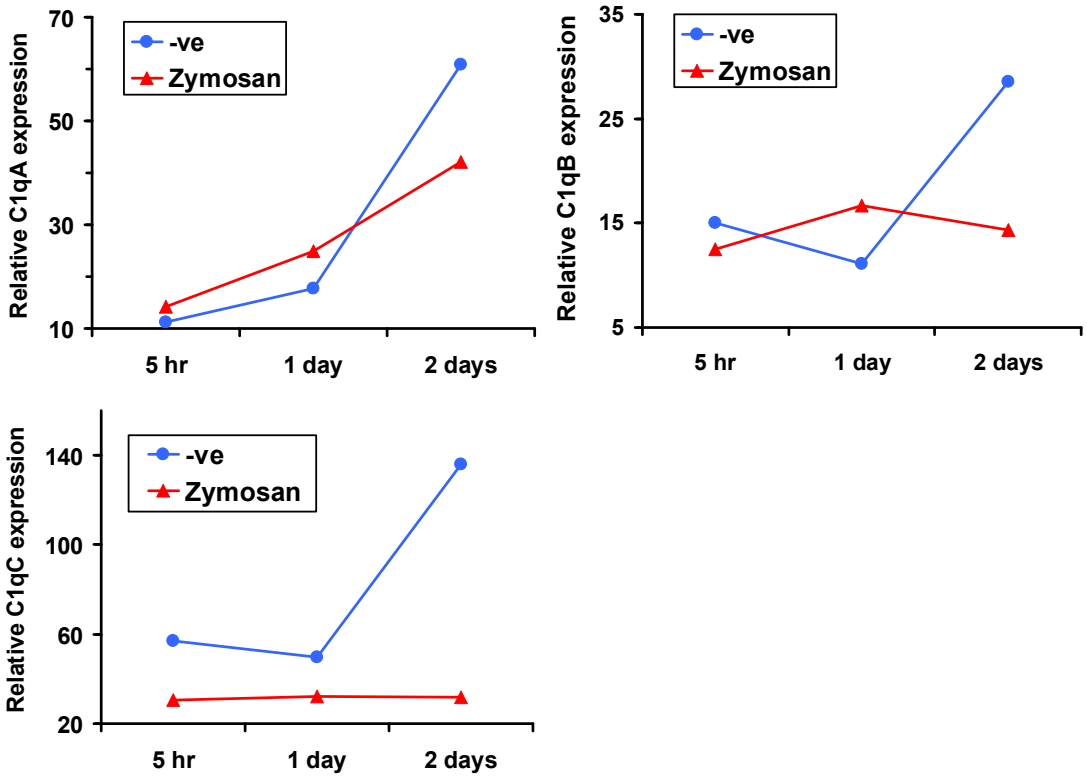

Figure 4.3. Quantitation of C1q mRNA in DC following zymosan treatment. 10^6 cells DCs were stimulated with zymosan or left unstimulated. Cells were harvested for RNA extraction over the 3 time-points. mRNA was converted to cDNA and analyzed via real-time PCR for the quantitation of the 3 C1q chains. The relative expression levels for all C1q chains were normalized to GAPDH.

The downregulation of C1q expression by zymosan appeared to be specific and not due to increased death of DCs. This was determined by measuring lactate dehydrogenase (LDH) enzyme released by dead cells into the culture media (Fig. 4.4). The levels of LDH release in all experiments were comparable with untreated DC (-ve).

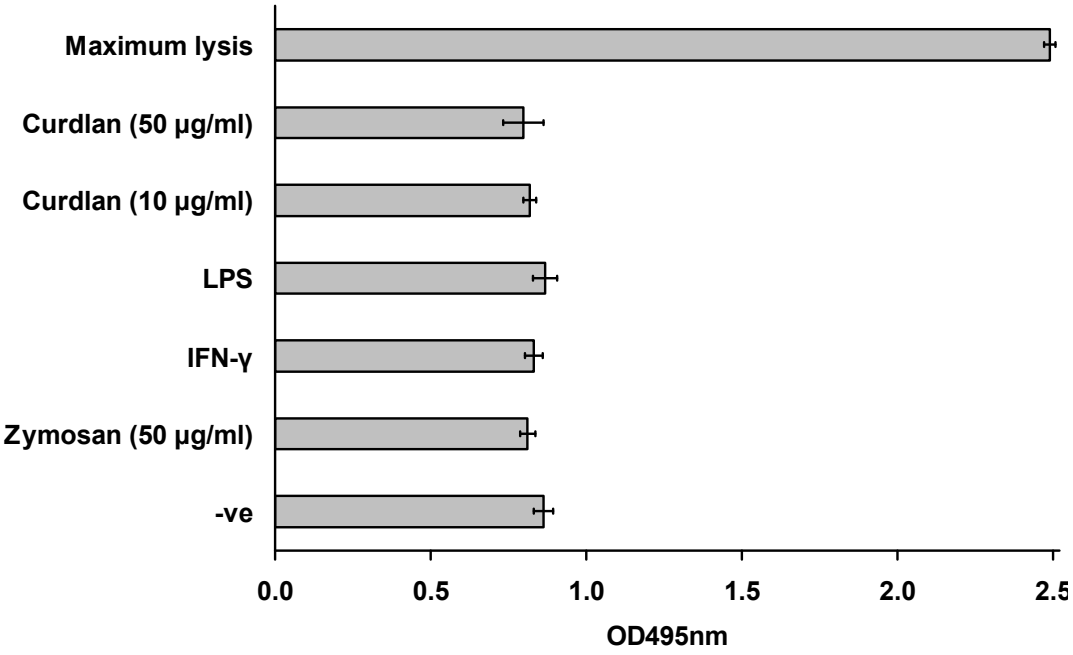

Figure 4.4 Determination of cell death in DCs after various treatments by measuring released lactate dehydrogenase (LDH). Cell culture supernatant was collected after cells were treated with different stimuli for 2 days. The level of LDH enzyme which is released upon cell death was measured as reflected in the graph as the OD495nm values. Maximum lysis indicates the maximum LDH released upon total cell lysis of unstimulated cells. Stimulations were performed in triplicates and all results are presented as means ± SD.

4.3 Neither opsonization of zymosan by serum factors nor its phagocytosis were required for C1q downregulation

Zymosan is a particulate extract of *S. cerevisiae* cell wall which is widely used to represent the signature stimulus from fungi. It can be opsonized by complement components and can therefore interact with immune cells either directly or after complement opsonization (Le Cabec *et al.*, 2002). In the experiments described so far, heat-inactivated bovine calf serum was used which should exclude the involvement by complements. To fully exclude any possible role played by complements, we stimulated DCs with zymosan in a serum-free media devoid of complements and other opsonins (Fig. 4.5A). This did not affect the downregulation of C1q production by zymosan, although the absolute amount of C1q produced was lower. It suggests that the inhibitory effect of zymosan probably involves direct zymosan interaction with DCs. The question remains if zymosan downregulation of C1q production requires its phagocytosis by DCs. However, when cytochalasin D was added to DCs, it showed no significant impairment of zymosan downregulation of C1q production (Fig. 4.5B). Cytochalasin D is an inhibitor of actin polymerization which inhibits phagocytosis.

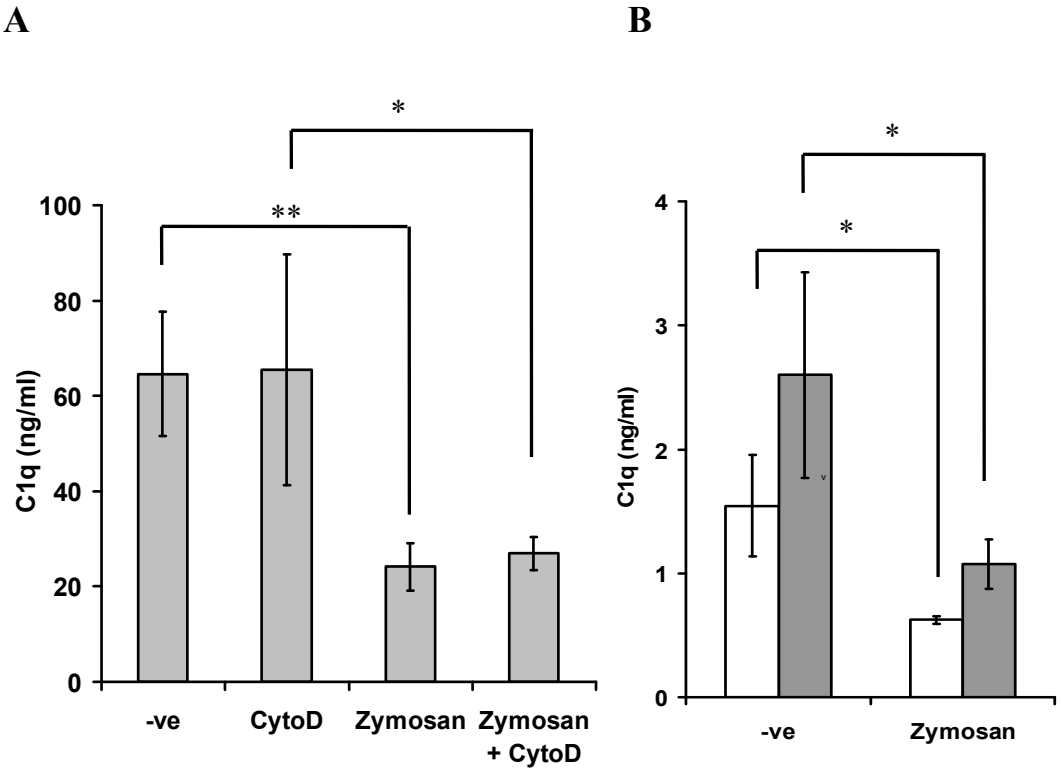

Figure 4.5. Neither serum factors nor phagocytosis are required for C1q downregulation by zymosan. (A) DCs were either untreated or pre-treated with cytochalasin D (1 μM), which inhibits phagocytosis, before stimulation with zymosan. Supernatant was collected after 2 days for analysis using ELISA. **(B)** The experiment was performed using serum-free CD-hybridoma medium (Gibco). Downregulation of C1q production by DCs after zymosan treatment is observed after 2 days (unshaded bars) and also after 2 additional days (grey bars). Generally, C1q production by DCs cultured in CD-hybridoma medium is greatly reduced. All stimulations were performed in triplicates and results are presented as means ± SD

4.4 Dectin-1 but not TLR signaling is required for zymosan downregulation of C1q production

Zymosan recognition by DCs is mediated through TLR2/6 and the C-type lectin Dectin-1. When zymosan is treated with hot alkali (termed depleted zymosan here), it removes moieties that stimulate the TLRs but remains potent in Dectin-1 stimulation (Ikeda *et al.*, 2008). To identify the contributions from TLRs and

Dectin-1 in downregulating C1q production, DCs were stimulated separately with zymosan and depleted zymosan. Both stimuli resulted in similar levels of suppression of C1q expression (Fig. 4.6A). This suggests that Dectin-1 is a novel receptor that can mediate the downregulation of C1q in DCs. We confirmed that Dectin-1 was expressed on the surface of DCs (Fig. 4.7). Curdlan is a pure β-glucan ligand for Dectin-1 and does not activate TLRs, thus it is a more specific activator for Dectin-1 (LeibundGut-Landmann *et al.*, 2007). Curdlan stimulation of DCs caused a downregulation of C1q production, similar to that caused by zymosan and the inhibition was also dose-dependent (Fig. 4.6B). We also confirmed via intracellular C1q staining that less C1q was detected inside DCs upon curdlan stimulation, similar to that observed with zymosan treatment (Fig. 4.8).

We then examined whether co-stimulation of DCs with other TLR ligands affects zymosan inhibition of C1q production, as zymosan itself could activate TLR2 and TLR6 in addition to Dectin-1 (Ozinsky *et al.*, 2000). An assortment of TLR ligands were used to co-stimulate DCs with depleted zymosan. None of these ligands affected C1q downregulation by depleted zymosan (Fig. 4.6C). Thus, zymosan downregulates C1q production by DCs through Dectin-1 stimulation and is independent of TLR engagement.

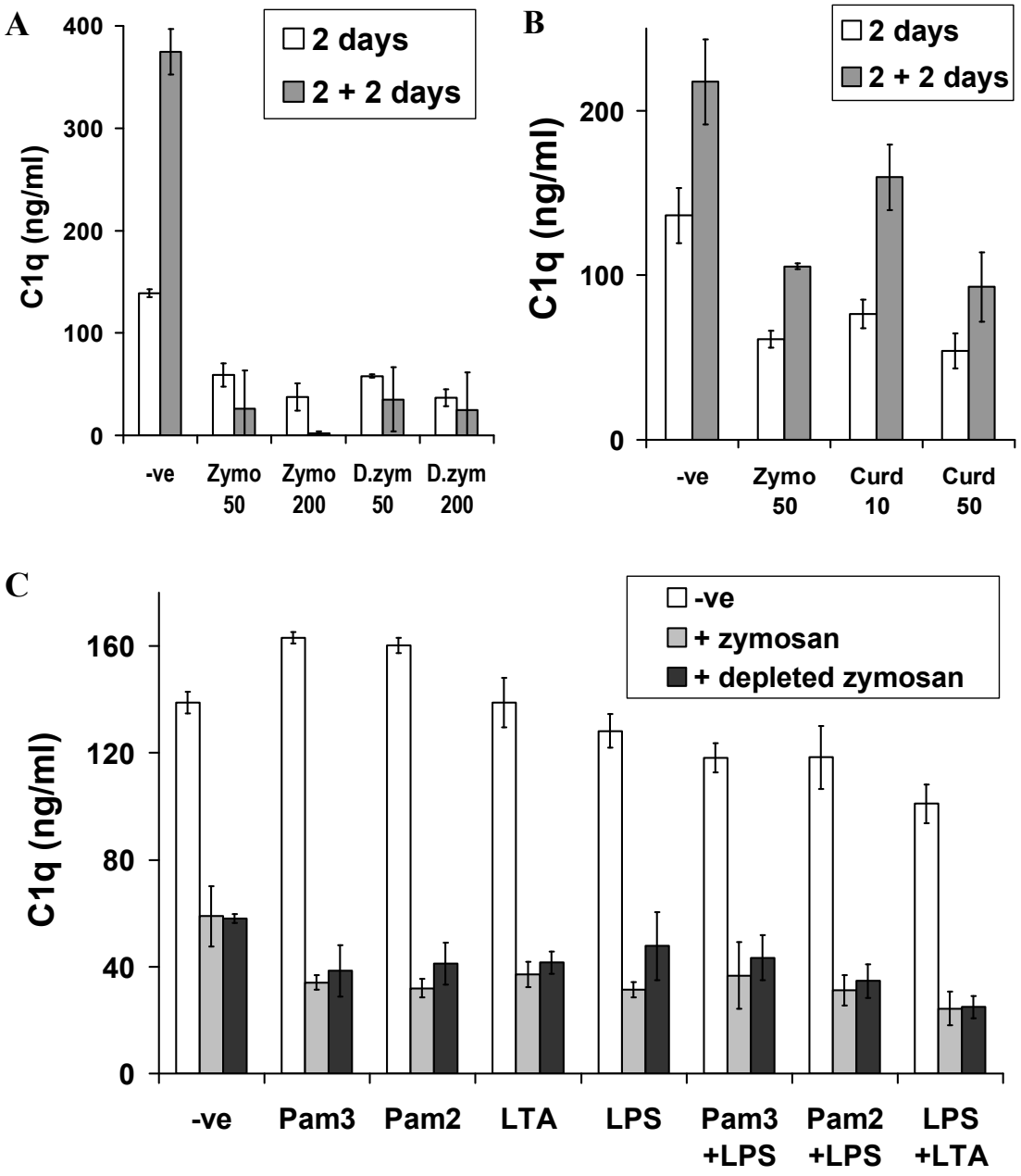

Figure 4.6. Zymosan signals through Dectin-1 and not TLRs to mediate downregulation of C1q production in DCs. (A) DCs were either treated with either 50 or 200 μg/ml of zymosan (zymo) or depleted zymosan (d.zym), which is devoid of TLR stimulatory ability but is still able to engage Dectin-1, or **(B)** curdlan, which is a specific Dectin-1 agonist. The dosage used was either 10 or 50 μg/ml. Supernatant was collected after 2 days and 2 + 2 days for analysis using ELISA. **(C)** DCs were left unstimulated or stimulated with zymosan or depleted zymosan together with various TLR ligands. Supernatant was collected after 2 days of culture and analyzed by ELISA. All experiments were performed in triplicates and the results are presented as means ± SD.

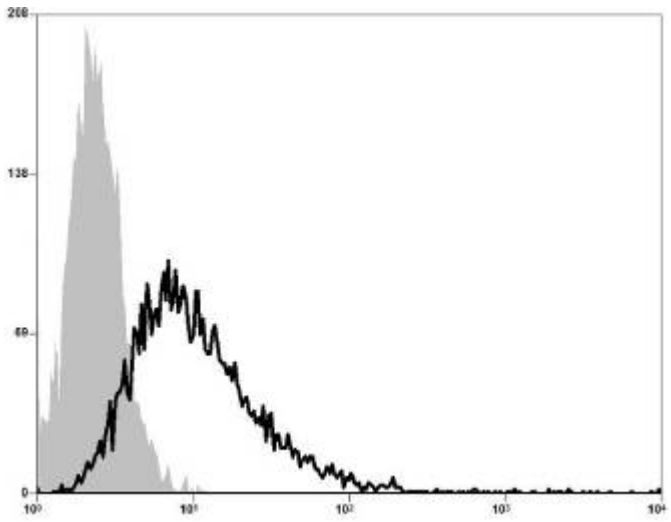

Figure 4.7. Dectin-1 is expressed on DC surface. DCs were harvested after 6 days of differentiation, stained with a monoclonal antibody specific against human Dectin-1 or an isotype control and counterstained with a secondary antibody. Cells were then fixed and analyzed by flow cytometry. Grey histogram – isotype control; Black histogram – Dectin-1.

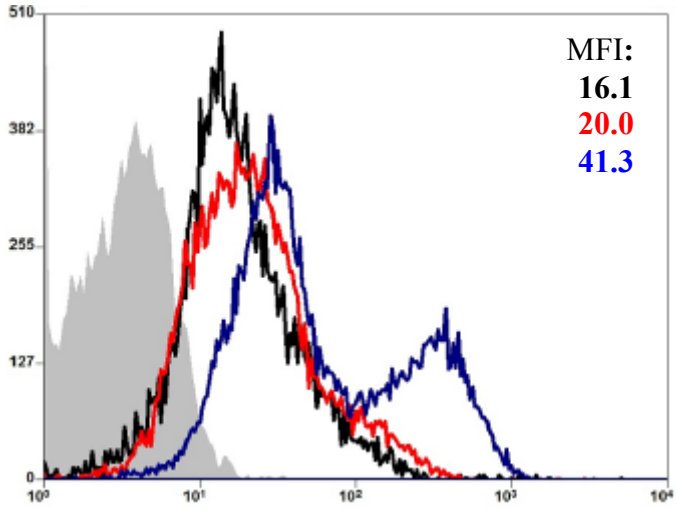

Figure 4.8. Reduction in intracellular C1q levels upon curdlan or zymosan treatment. DCs were treated for 4 days with zymosan or curdlan at 50 μg/ml, fixed, permeabilized and stained with an anti-C1q antibody or isotype control, and then a PE-conjugated goat-anti mouse IgG was used to visualize the C1q staining. Grey histogram – isotype control; Black histogram – curdlan; Red histogram – zymosan, Blue histogram – untreated. MFI values are the mean fluorescent intensities of the respective histograms.

4.5 Dectin-1 inhibition of C1q production can suppress the IFN-γ enhancement of C1q

We have shown in Chapter 3.4 that IFN-γ showed the opposite effect on C1q expression as Dectin-1 signaling, whereby it enhanced C1q production. Thus we wanted to determine if the suppressive effects of Dectin-1 signaling could overcome the C1q enhancing signals of IFN-γ or the reverse.

The results Fig. 4.9A show the expected results after 2 days of DC stimulation, where zymosan and curdlan treatment suppressed C1q production and in contrast, IFN-γ enhanced C1q production. When zymosan or curdlan was added together with IFN-γ to DCs and cultured for 2 days, C1q production was suppressed down to the levels of zymosan or curdlan stimulation alone. This suggests that Dectin-1 inhibition of C1q production is a powerful signal and can overcome the C1q upregulating effects of IFN-γ.

In the same experiment, some cells were primed with zymosan, curdlan or IFN-γ alone, or zymosan + IFN-γ, or curdlan + IFN-γ, or left unprimed. After 2 days, the cells were washed and then stimulated with zymosan, curdlan, IFN-γ or left unstimulated and are labeled as (af) in Fig. 4.9B. These cells were then cultured for 2 more days and the C1q levels secreted into culture supernatant was quantitated. C1q secretion by cells that were unprimed or primed with zymosan, curdlan or IFN-γ, washed and then received no further stimulation (-ve (af)) followed the same profile as cells in Fig. 4.9A.

When cells that were primed with IFN-γ for 2 days and then treated either with zymosan (zymo(af)) or curdlan (curd(af)), less C1q was produced than in cells primed with IFN-γ and left untreated. Cells primed with zymosan and then stimulated with IFN-γ (IFN-γ (af)) showed no enhancement in the C1q levels. But in contrast, cells primed with curdlan and then similarly received IFN-γ stimulation had higher C1q secretion than cells primed with curdlan alone and left unstimulated, although the levels are still well below that of the unstimulated control.

Cells that were unprimed for 2 days, and then stimulated with zymosan or curdlan still exhibited suppression in their C1q production. When the same unprimed cells were stimulated with IFN-γ after 2 days, C1q production was still enhanced.

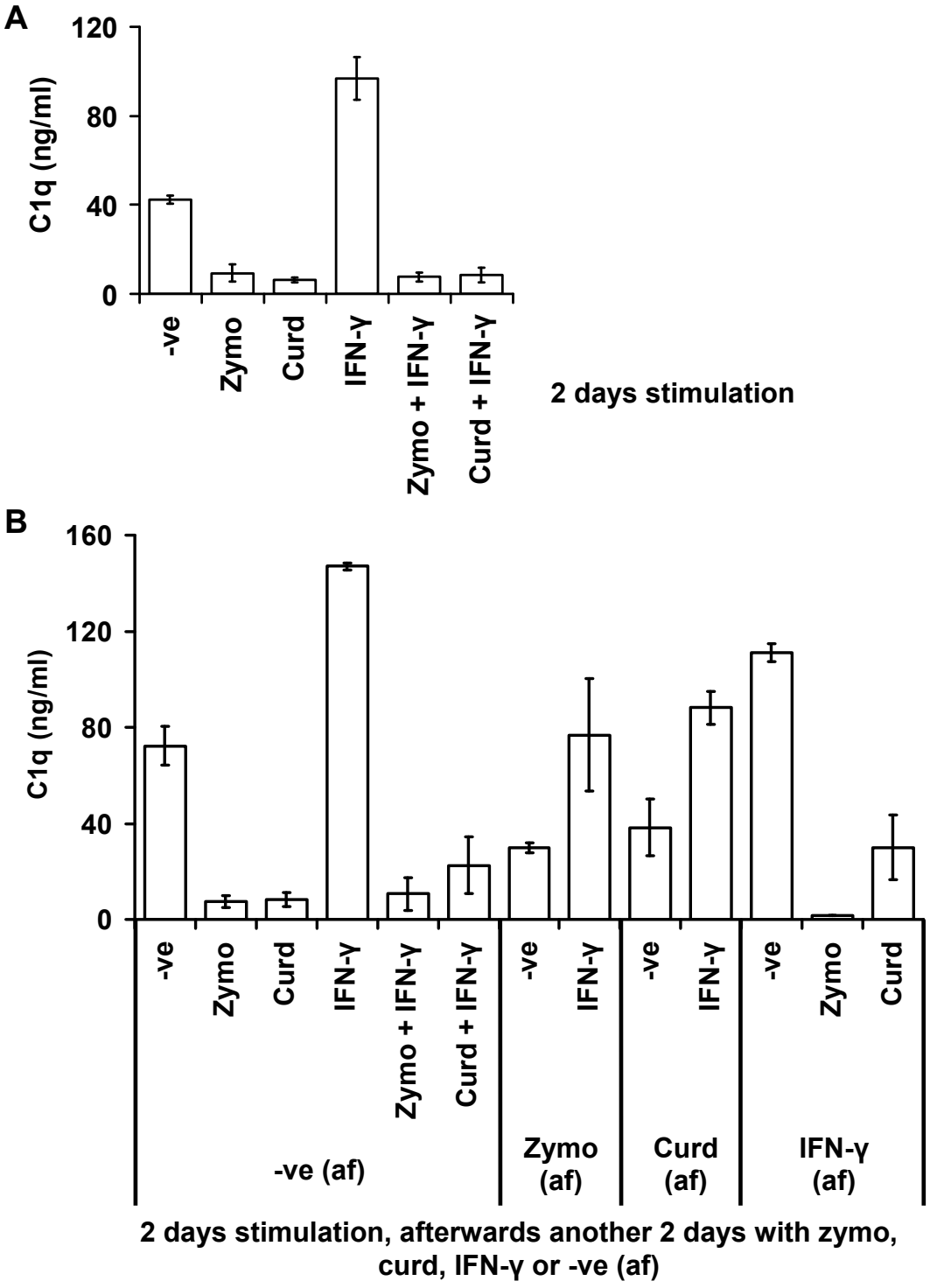

Figure 4.9. Dectin-1 stimulation overcomes the enhancement of C1q production by IFN-γ. DCs were stimulated with the Dectin-1 ligands zymosan and curdlan (50 μg/ml for both) or IFN-γ (100 ng/ml) alone or together for **(A)** 2 days, or **(B)** after 2 days, the cells were washed and cultured for 2 more days without the addition of further stimulation, -ve (af); or with zymosan, Zymo (af); or with curdlan, Curd (af); or with IFN-γ, IFN-γ (af). C1q levels were analyzed by ELISA in all DC culture supernatant. Experiments were performed in triplicates and the results are presented as means ± SD.

4.6 Dectin-1 induced downregulation of C1q production does not signal through Syk

Ligand binding of of Dectin-1 causes the ITAM domains to be phosphorylated by Src kinases which recruits Syk and forming a complex comprising of caspase recruitment domain 9 (CARD9), BCL10 and MALT1 (Hara *et al.*, 2007). This leads to activation of NF-κB and production of cytokines following Dectin-1 activation. We asked whether inhibiting Syk can abrogate the inhibitory effects of Dectin-1 activation. A Syk inhibitor piceatannol was to treat DCs at various dosages before the addition of curdlan. This inhibitor was unable to abrogate the inhibitory effects of curdlan, although at higher dosages it slightly inhibited C1q production in general (Fig. 4.10).

To confirm that piceatannol is functional, we measured the levels of the cytokines IL-6 and IL-10 produced in the culture supernatant following piceatannol treatment. Curdlan stimulation of cells resulted in the production of IL-6 and IL-10 (Fig. 4.11A and B). Piceatannol treatment inhibited IL-6 levels by about one-quarter while IL-10 levels were reduced by more than half. This indicates that piceatannol did indeed have inhibitory effects, which worked in inhibiting cytokine production following Dectin-1 stimulation, but showed no effects in subjugating the inhibitory effects on C1q production. Thus far the results suggest that Syk signaling downstream of Dectin-1 is not involved in controlling the downregulation of C1q production.

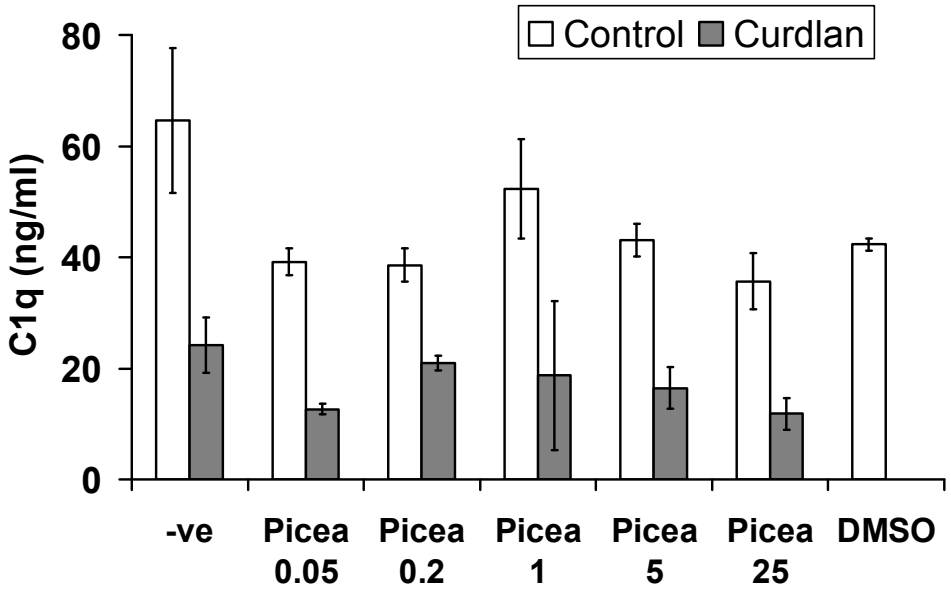

Figure 4.10. Inhibition of Syk does not restore C1q levels downregulated upon curdlan treatment back to unstimulated levels. Various concentrations of piceatannol (in μM), the inhibitor of Syk were used to pre-treated DCs before stimulation with curdlan. DMSO was added as a vehicle control. Supernatant was collected after 2 days for C1q analysis using ELISA. Stimulations were performed in triplicates and results are presented as means ± SD.

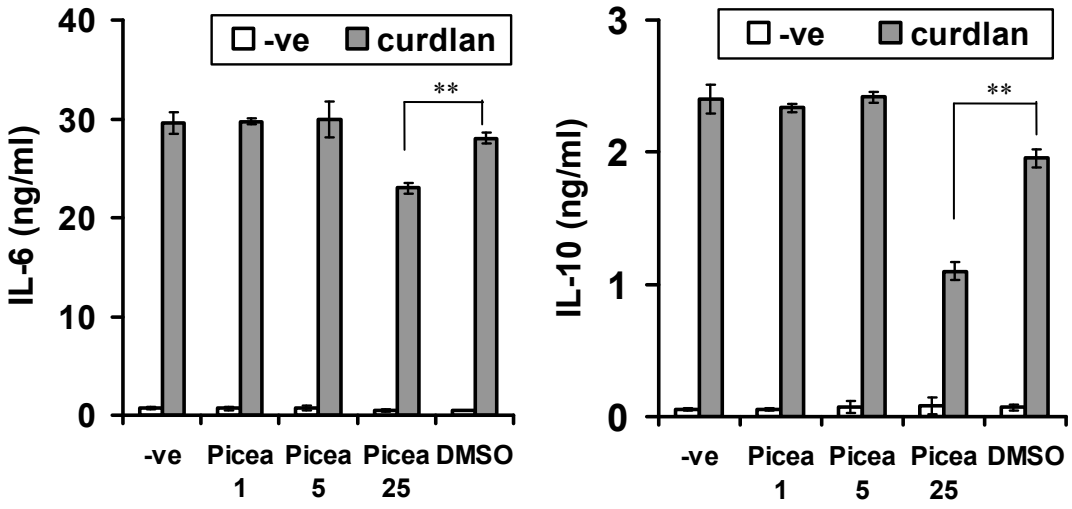

Figure 4.11. The Syk inhibitor piceatannol attenuates production of IL-6 and IL-10 after Dectin-1 activation. Culture supernatant from the experiment in Fig. 4.10 was assayed for IL-6 **(A)** and IL-10 **(B)** production to determine if piceatannol could inhibit Syk activation following Dectin-1 ligation with curdlan. Stimulations were performed in triplicates and results are presented as means ± SD.

4.7 Arachidonic acid release and ROS generation is not coupled to the downregulation of C1q production on Dectin-1 stimulation

Cytosolic phospholipase A_2 ($cPLA_2$) is also activated by zymosan in macrophages and this leads to the release of arachidonic acid and production of eicosanoids, which initiate acute inflammation (Suram *et al.*, 2006). So far, no report has shown that arachidonic acid regulates C1q expression and thus we asked if this could be the mechanism behind the observed downregulation of C1q production after Dectin-1 activation.

We used a specific inhibitor of $cPLA_2$, pyrrolidine-2 (Suram *et al.*, 2006), during curdlan stimulation of DCs. The inhibitor alone without curdlan stimulation caused a slight increase in C1q production at 2 μM and C1q production was further increased when it was used at 10 μM (Fig. 4.12). Nevertheless, it showed no suppression of curdlan inhibition of C1q production in DCs. It suggests that $cPLA_2$ mediated arachidonic acid release, which was induced by curdlan through Dectin-1, was not responsible for Dectin-1 induced inhibition in C1q production.

Signaling by Dectin-1 in macrophages also triggers ROS production (Underhill *et al.*, 2005). We then investigated if ROS is inhibiting C1q production. This was examined by adding diphenylene iodonium (DPI), an inhibitor of the NADPH oxidase and nitric oxide synthase (Riganti *et al.*, 2004), to DCs during the 2 days of curdlan stimulation. At a low concentration (0.05 μM), DPI alone without curdlan slightly increased C1q production. However, it did not affect the curdlan inhibition of C1q production (Fig. 4.12). At higher concentrations (0.2 μM and 1 μM), it inhibited C1q production but again it had no effect on the inhibitory effects of

curdlan. Thus, ROS production, which is expected to be induced by curdlan, does not mediate the downregulation of C1q Dectin-1 ligation.

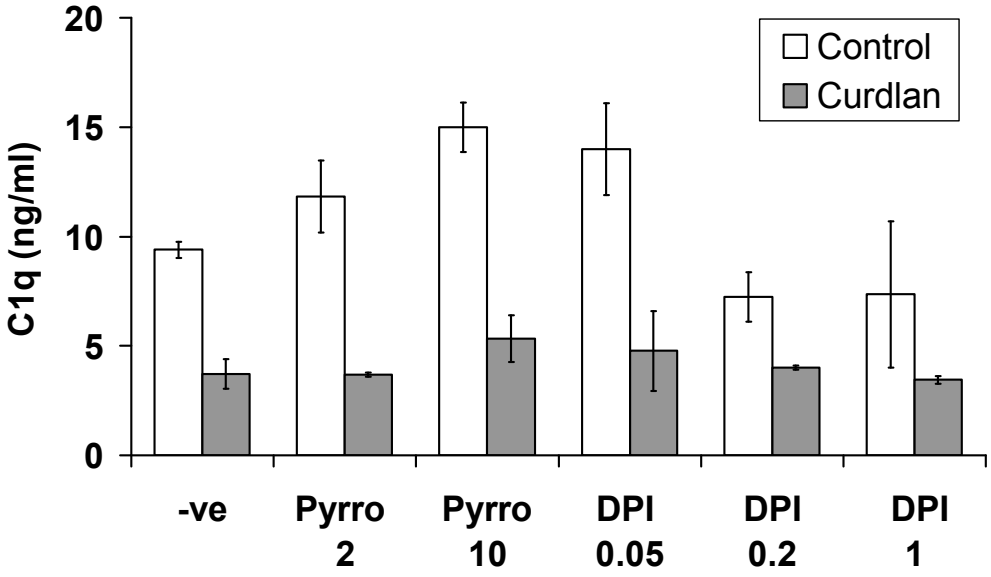

Figure 4.12. Neither arachidonic acid nor ROS release following Dectin-1 ligation cause the downregulation of C1q in DCs. DCs were pre-treated with pyrrolidine-2, an inhibitor of cytosolic phospholipase A_2 that generates arachidonic acid, or diphenyleneiodonium chloride (DPI), which inhibits the processes leading to reactive oxygen species production, at the indicated dosages (in μM) before curdlan was added. Cells were stimulated for 2 days and supernatant was analyzed for C1q production by ELISA. Stimulations were performed in triplicates and results are presented as means ± SD.

4.8 Involvement of both Raf-1 and Ca^{2+} signaling are excluded from the suppression of C1q production following Dectin-1 activation

Raf-1 signaling is triggered downstream of Dectin-1 activation (Gringhuis *et al.*, 2009), and whether it leads to suppression of C1q production was examined. We used the same system of treating DCs with a specific inhibitor; in this case we used the Raf-1 inhibitor GW5074, prior to stimulation with curdlan. C1q secretion by DCs into the culture supernatant was then assayed by ELISA. Treatment of DCs

with low dosages of GW5074 (2 μM and 10 μM) did not restore C1q levels after curdlan stimulation to the levels of unstimulated cells (Fig. 4.13). A higher dosage of 25 μM slightly reduced C1q secretion by unstimulated DCs, but nevertheless there was still no abrogation of the inhibitory effects of curdlan.

Dectin-1 stimulation elicits Syk-mediated activation of intracellular Ca^{2+} flux (Xu *et al.*, 2009b). This leads to the production of many cytokines including IL-2, IL-6, IL-10, IL-12, IL-23, and TNF-α. To investigate whether Ca^{2+} signaling was involved in Dectin-1-mediated inhibition of C1q production, DCs were pre-treated with a cell permeable Ca^{2+} chelator BAPTA-AM before stimulation with curdlan. A total of 3 different concentrations were tested but all treatments failed to affect the downregulation of C1q levels following curdlan stimulation (Fig. 4.13).

We also analyzed IL-6 and IL-10 levels in the culture supernatant to determine if the inhibitors were functional. GW5074 treatment at 25 μM inhibited curdlan-induced IL-6 and IL-10 levels to about ¾ that of non-treated levels (Fig.4.14A and B). BAPTA-AM treatment at 10 μM only managed to reduce IL-6 levels by about 10% but IL-10 levels were reduced by about ¼.

These results suggest the exclusion of another two mechanisms, the Raf-1 and Ca^{2+} signaling, from being involved as the effectors downstream of Dectin-1 signaling in downregulating C1q expression.

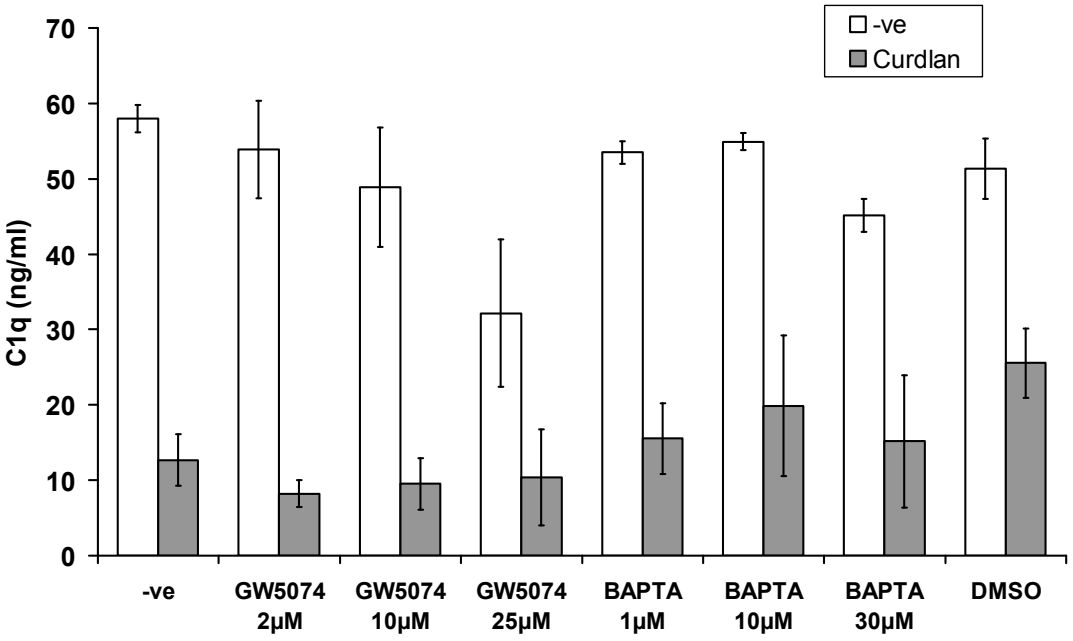

Figure 4.13. The inhibition of Raf-1 or Ca^{2+} influx inhibition could not abrogate the inhibitory effects on C1q production after Dectin-1 ligation. DCs were pre-treated with the Raf-1 inhibitor GW5074 or intracellular Ca^{2+} chelator BAPTA-AM at the indicated dosages before curdlan was added. Cells were stimulated for 2 days and supernatant collected and analyzed by ELISA. Stimulations were performed in triplicates and results are presented as means ± SD.

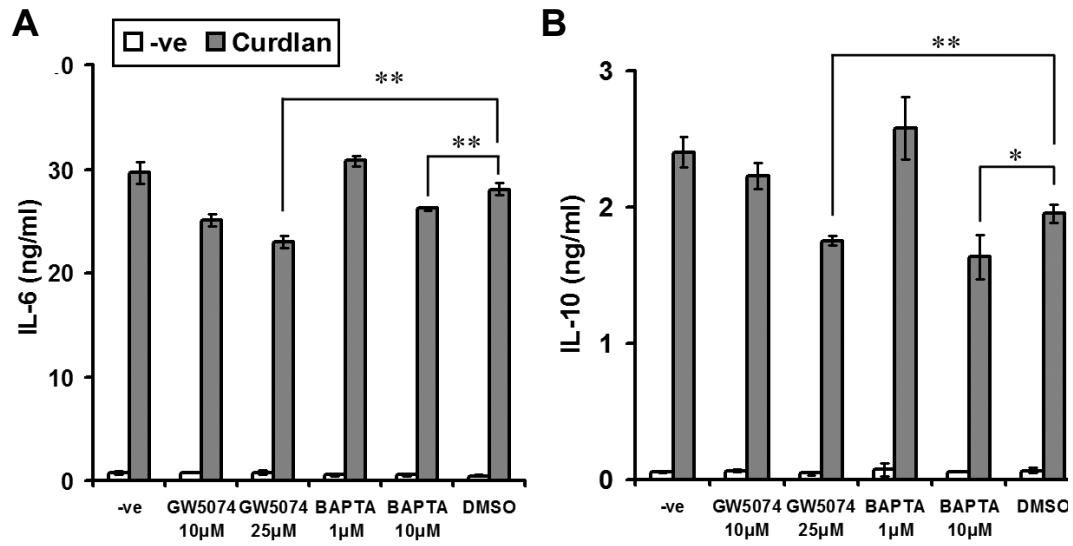

Figure 4.14. Raf-1 inhibitor GW5074 and Ca^{2+} chelator BAPTA-AM partially attenuates the production of IL-6 and IL-10 after Dectin-1 activation. Culture supernatant from the experiment in Fig. 4.13 was used to assay for IL-6 **(A)** and IL-10 **(B)** to determine if GW5074 and BAPTA-AM could inhibit cytokine production following Dectin-1 stimulation with curdlan. Stimulations were performed in triplicates and results are presented as means ± SD.

Chapter 5 Regulation of DC production of C1q by IFN-α and IFN-γ – Linkage to SLE pathogenesis

5.1 Introduction

ICs cause unabated IFN-α production in SLE patients, especially during disease flares (Pascual *et al.*, 2006). The main cellular source of IFN-α are the pDCs activated by ICs. pDC numbers are reduced in SLE blood (Blanco *et al.*, 2001), but this is pathogenic as these cells massively infiltrate inflammed lupus skin (Farkas *et al.*, 2001). IFN-α contributes to increased autoantibody secretion and immune complex formation in SLE by directly activating B cells and differentiating mature B cells into antibody-producing plasma cells (Jego *et al.*, 2005). IFN-α can directly induce inflammatory DC differentiation and maturation, and possibly activating autoreactive T lymphocytes that have escaped deletion during central tolerance (Blanco *et al.*, 2005). Mature DCs generated from IFN-α exposure can activate cytotoxic $CD8^+$T cells to lyse cells and release nucleosomes that can be captured and presented by DCs as autoantigens.

In order to understand how the protective role of C1q is connected to the pathogenic role of type I IFN, we examined how IFN-α and IFN-γ regulate C1q production in DCs. There is no data so far whether IFN-α can modulate C1q production. We included IFN-γ in this study because there are earlier studies reporting that it increases or decreases C1q production in macrophages in different studies.

5.2 C1q production by DC is attenuated by prolonged IFN-α treatment

Elevated serum IFN-α level is commonly detected in SLE patients (Kim *et al.*, 1987). SLE patients have chronically elevated serum levels of IFN-α during disease flares. Since C1q deficiency is associated with SLE development, whether IFN-α regulates C1q production by DCs is relevant to SLE pathogenesis. Therefore, we investigated if these two factors are intimately regulated.

We established a system that addresses both short-term and chronic exposure of cells to the IFN-α inflammatory signals. We initially stimulated DCs for 48 hr with two concentrations of IFN-α (200U/ml and 5000U/ml, respectively labelled as IFNa(1) and IFNa(2) in the figures), but this short-term stimulation caused no detectable change in C1q production as determined by ELISA (Fig. 5.1A).

After 48 hr, the IFN-α treated DCs were washed and cultured for a further 48 hr without any further stimuli (Fig. 5.1B, neg (af)) or with IFN-α (Fig. 5.1B, IFNa (2) (af)). This time, we observed a decline in C1q production in both cases. In the 96 hr culture, C1q production was decreased by about half in the IFN-α-primed DCs compared to the untreated DCs even though IFN-α was absent during the second 48 hr. When IFN-α was used to prime the DCs in the first 48 hr, washed and added again in the second 48 hr, a marked decreased in C1q production was observed, whereby the C1q levels decreased by about ¼ compared to DCs that were initially unprimed and then IFN-α-treated for the subsequent 48 hr. These results show that IFN-α only significantly inhibits C1q production in DCs upon prolonged treatment, mimicking chronic IFN-α production in SLE.

5.3 IFN-γ enhances C1q production and also abrogates IFN-α inhibition

In Chapter 3.4, IFN-γ was found to have opposite effects compared to IFN-α in C1q regulation, in that it upregulated C1q production over the 2 days and 2 + 2 days culture. Thus, we decided to further study how IFN-γ increases C1q production in DCs.

Firstly, IFN-γ stimulation alone primed DCs to produce more C1q in the first 48 hr (Fig. 5.1A), and also after the cells were washed and cultured for another 48 hr (Fig. 5.1B, -ve (af)) when compared to unprimed cells. Secondly, with both IFN-α and IFN-γ co-stimulation, DCs still produced high levels of C1q, equal to that of IFN-γ alone (Fig. 5.1A). In this same co-stimulation of DCs, IFN-α was unable to prime DCs so that less C1q was produced in the second set of 48 hr culture (Fig. 5.1B, -ve (af)). Thirdly, IFN-γ has the ability to restore C1q production in IFN-α-primed DCs (Fig. 5.1B, IFN-γ (af) for cells treated with IFNa (1) and IFNa (2)). This shows that IFN-γ is of an antagonistic nature to the inhibitory effects associated with IFN-α, and is dominant over IFN-α in this context.

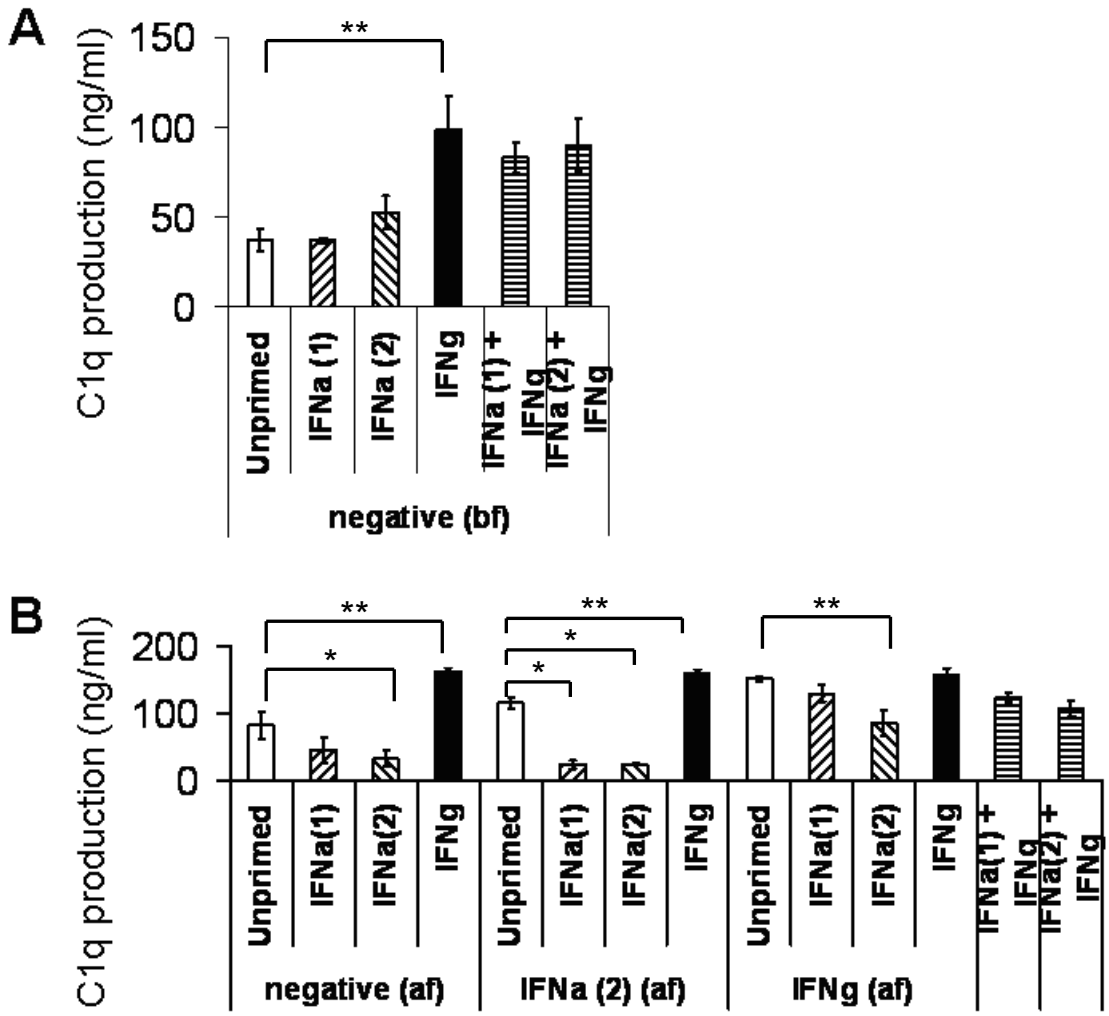

Figure 5.1. Distinct and antagonistic regulation of C1q production by IFN-α and IFN-γ. (A) DCs were cultured from monocytes using GM-CSF and IL-4 and, at day 6, the cells were treated with IFN-α at 200 U/ml (1) or 5000 U/ml (2) or IFN-γ (100 ng/ml) for 48 hr, or left unprimed. `bf', before further treatment. C1q production was measured by ELISA **(B)** After washing, the cells were then treated with IFN-α (2) or IFN-γ or left untreated for 48 hr. `af', after initial treatment for 48 hr. C1q production was measured by ELISA. All experiments were performed in triplicates and results are presented as means ± SD.

5.4 Decreased C1q secretion following IFN-α treatment is not associated with increased DC death

The IFN-α-mediated downregulation of C1q expression appeared specific and was not due to increased death of DCs. We measured LDH enzyme secretion into the culture media following the treatment of DCs with both IFN-α and IFN-γ for 2 days and 2 + 2 days. (Fig. 5.2A). The levels of LDH release in all experiments were comparable with untreated DCs (-ve) for both time-points.

In addition to measuring the LDH secretion, we also checked the viability of cells by propidium iodide (PI) staining. DCs were left unstimulated or stimulated with IFN-α and IFN-γ for 2 days, washed and then cultured for another 2 days. Cells were harvested at both time-points, stained with PI and analyzed immediately using flow cytometry. Cells staining positive for PI are non-viable and have disrupted membranes. For 2 days stimulation (Fig. 5.2B), non-treatment and IFN-α treatment resulted in 28% of DCs staining for PI while IFN-γ treatment actually resulted in 37% of cells being PI positive. This was also consistently observed for the 2 + 2 days cultures, whereby IFN-γ treatment resulted in the highest cell death of 26%, and IFN-α gave the lowest cell death percentage of 18% (Fig. 5.2C). For untreated cells, 24% were stained PI positive.

Thus, IFN-α treatment does not downregulate C1q by increasing DC death, in fact it seems that DCs actually survive slightly better following IFN-α stimulation.

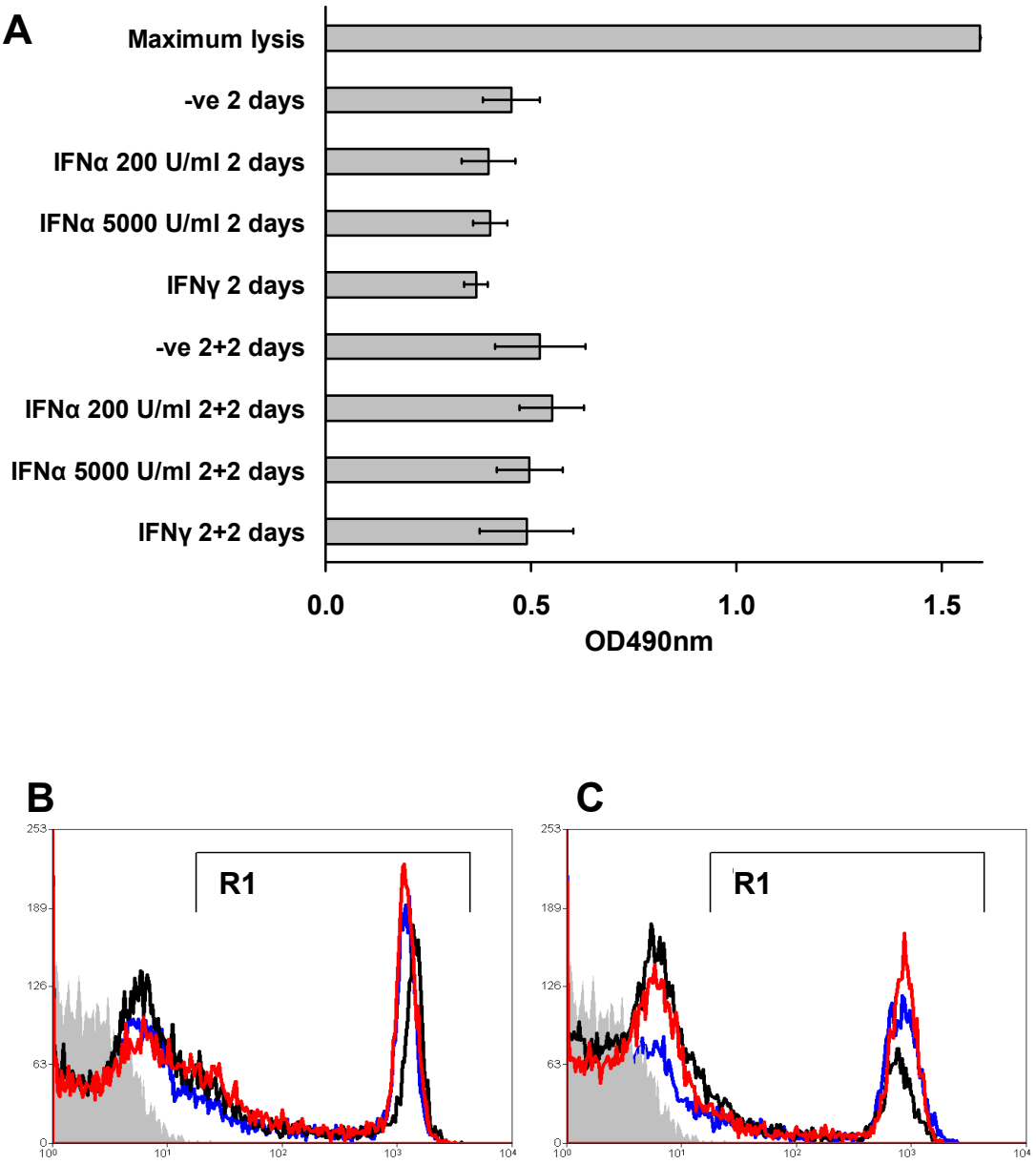

Figure 5.2. Reduction in C1q secreted after IFN-α treatment is not due to increased cell death. (A) Lactate dehydrogenase levels were measured from supernatant of DCs stimulated with IFN-α, IFN-γ or left unstimulated for 2 days and 2 + 2 days. Maximum lysis indicates the maximum levels of LDH released upon total cell lysis of unstimulated cells. DCs stimulated with IFN-α, IFN-γ or left unstimulated for **(B)** 2 days and **(C)** 2 + 2 days were harvested, washed, counterstained with the vitality dye propidium iodide (PI) and analyzed via flow cytometry immediately. Blue – Unstimulated cells; Black – IFN-α; Red – IFN-γ; Grey histogram – no PI staining. These are the percentage of PI positive cells in the region R1 for 2 days (B): -ve – 28%; IFN-α – 28%; IFN-γ – 37% and 2 + 2 days (C): -ve – 24%; IFN-α – 18%; IFN-γ – 26%.

5.5 Downregulation of secreted C1q protein by chronic IFN-α stimulation does not occur at the transcriptional level

We next examined the expression of C1q in DCs at the transcriptional level following IFN-α as well as IFN-γ treatments. Initially, IFN-α does not appear to affect the levels of all three C1q subunit mRNA up to 24 hr after stimulation. At 48 hr, DCs treated with IFN-α upregulated all three C1q subunit mRNA (about 3-fold for C1qA and C1qB, and about 5-fold for C1qC, Fig.5.3, A-C) compared to untreated cells. At the maximum time-point assayed of 96 hr, mRNA of C1q subunits A, B and C were all significantly increased after prolonged treatment with IFN-α compared to untreated cells. This differs significantly from the ELISA analysis which showed that 96 hr of priming with IFN-α significantly inhibited the levels of secreted C1q protein. This discrepancy warrants further investigation into whether IFN-α stimulation of DCs affects C1q production at the translational level or its secretion.

We also examined how IFN-γ regulates C1q expression in DCs at the transcriptional level by stimulating these cells with IFN-γ for 5, 24, 48 and 96 hr. All three C1q subunit mRNA species were increased by IFN-γ noticeably after 24 hr, and continued to increase in a time-dependent manner until 96 hr (Fig. 5.3, A-C).

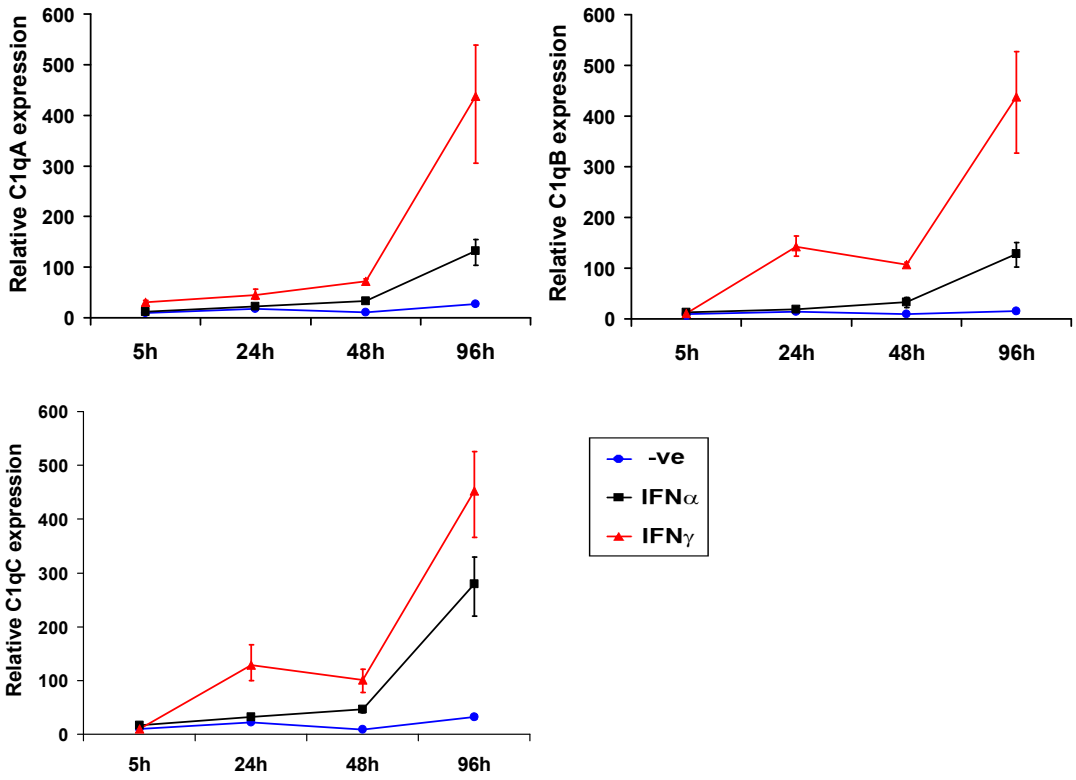

Figure 5.3. IFN-α surprising increased C1q mRNA production in DCs together with IFN-γ. DCs were stimulated with IFN-α (200 U/ml; black), IFN-γ (100 ng/ml; red) or left untreated (blue) for 5 hr, 24 hr and 48 hr. The cells were then washed and cultured for another 48 hr to make up to 96 hr of total culture time. This mimics the system used in Fig. 5.1 to analyze secreted C1q protein in culture supernatant after prolonged stimulation of DCs with IFN-α. Total RNA were isolated for each time-point, converted to cDNA and subjected to real-time PCR detection of all 3 chains of C1q. mRNA levels were normalized to GAPDH and the C1q expression level of the 5 hr untreated DCs was calibrated to 1.

5.6 The downregulation of C1q after chronic IFN-α stimulation is also not regulated at the protein translational level

As C1q mRNA levels were enhanced with both IFN-α and IFN-γ stimulation, we investigated if this led to a corresponding increase in the amount of C1q protein produced. Indeed the amount of C1q detected from Western blots of DC lysates corresponded to that of the mRNA levels (Fig. 5.4A). At every time-point, DCs stimulated with IFN-γ had the highest levels of C1q, followed by DCs stimulated with IFN-α and unstimulated DCs expressed the lowest levels of C1q. Generally the C1q levels increased in a time-dependent manner for all treatments.

As a further confirmation, we detected the expression of the full C1q protein using intracellular flow cytometry after the DCs were stimulated for 2 + 2 days. The results corroborates the results observed for the real-time PCR and Western blot data, in that unstimulated cells expressed the least C1q and IFN-γ treated cells showed the highest expression, with C1q expression level of IFN-α stimulated cells in between unstimulated and IFN-γ stimulated DCs (Fig. 5.4B).

Insofar, chronic IFN-α stimulation of DCs does not seem to reduce its transcription and translation of C1q. Instead, DCs stimulated with IFN-α actually expressed more C1q mRNA and protein. This suggests that IFN-α actually inhibited C1q secretion from DCs.

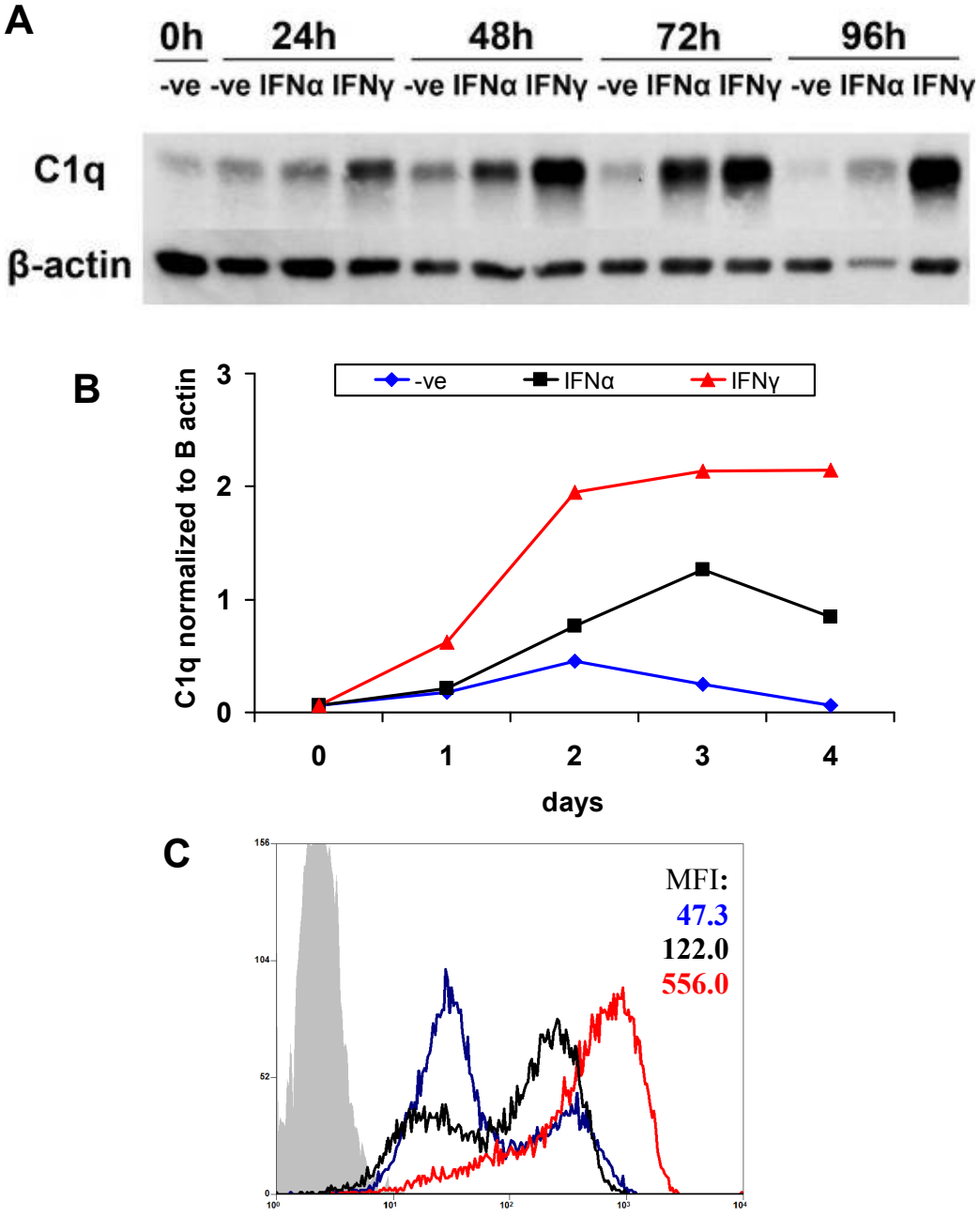

Figure 5.4. Intracellular C1q detection in IFN-α and IFN-γ stimulated DCs via Western blot and flow cytometry. (A) 5 X 10^5 DCs were stimulated with IFN-α, IFN-γ or were left unstimulated, and the cells were harvested and lysed at the time-points indicated. About 20 µg of each cell lysate was resolved via SDS-PAGE, Western blotted and probed for C1q with a goat polyclonal anti-C1q antibody. The membrane was stripped and re-probed with an anti-β-actin antibody. **(B)** Densitometric analysis of C1q levels in Fig.5.4A normalized to β-actin levels. **(C)** 5 X 10^5 DCs were stimulated with IFN-α, IFN-γ or left unstimulated for 2 days, washed and cultured for 2 more days. The cells were then harvested, fixed and permeabilized. Intracellular C1q was detected using a mouse anti-C1q antibody or isotype control and a PE-conjugated goat-anti mouse IgG was used to visualize the C1q staining. Grey histogram – isotype control; Blue histogram – no stimulation; Black histogram – IFN-α; Red histogram – IFN-γ.

5.7 C1q is mainly trapped in the endoplasmic reticulum and not transported to the Golgi apparatus for secretion after IFN-α stimulation

C1q synthesis was increased in DCs following IFN-α stimulation, but its secretion seems to be defective when compared to cells stimulated with IFN-γ. Proteins are exported out from cells via the secretory pathway. Newly synthesized proteins in the cytosol that are destined for secretion are translocated into the endoplasmic reticulum (ER), then packaged into vesicles which fuse to become and merge to form the *cis*-Golgi network (CGN). The proteins then moves through the Golgi to the terminal *trans*-Golgi network (TGN). Here, proteins are membrane-bound and secreted as vesicles (van Vliet *et al.*, 2003). Thus, if C1q secretion is defective, the C1q could possibly be trapped in the ER and unable to translocate to the Golgi network, or could be stuck within the Golgi apparatus and not be packaged into secretory vesicles.

We thus sought to pinpoint the localization of C1q within the intracellular compartments after IFN-α/IFN-γ treatment of DCs. C1q was stained together with the markers calreticulin (CRT) that localizes to the ER (Ramos *et al.*, 2007) and Syntaxin-16 (Syn16) that is found in the Golgi apparatus (Tang *et al.*, 1998). Proteins that are endocytosed at the plasma membrane are transported to the early endosome, of which the early endosome antigen-1 (EEA-1) is a marker (Mukherjee *et al.*, 2006). We stained for early endosome as a negative control, as C1q is produced internally by the cells and not taken in by endocytosis. Thus C1q should not co-localize with EEA-1.

High levels of C1q expression were detected in DCs stimulated with IFN-γ for 2 days (Fig. 5.5) and 2 + 2 days (Fig. 5.6), and most of the C1q were not localized in the ER. In fact, C1q were mostly found in the Golgi apparatus (Fig. 5.7 and 5.8) and this suggests that the C1q are ready to be packed for secretion. More C1q was detected after both 2 days and 2 + 2 days of IFN-α stimulation than in unstimulated cells. Strikingly, we found that most of the C1q were localized in the ER (Figs. 5.5 and 5.6). Only a small amount of C1q was detected in the Golgi apparatus after 2 days of IFN-α stimulation, and after 2 + 2 days of stimulation with IFN-α, more C1q now localize to the Golgi apparatus for secretion, but it is still noticeably lesser than in DCs stimulated with IFN-γ. Although C1q expression in unstimulated DCs was generally low, we observed that in cells that expressed C1q, the C1q appeared as distinct punctate bodies separate from the ER for both stimulation time-points (arrows for –ve DCs in Figs. 5.5 and 5.6). Some C1q in the unstimulated cells were seen to localize to the Golgi apparatus although in general, their expression levels were low (arrows for –ve DCs in Figs. 5.7 and 5.8). Co-staining of C1q with the early endosome marker EEA-1 shows no co-localization of the 2 signals for all stimulations and at both time-points (Figs. 5.9 and 5.10). This shows that C1q was produced intracellularly by the DCs, and that confocal microscopy can exclude compartments that C1q is not located in.

In summary, although IFN-α treatment increased C1q expression, its secretion mechanism was impaired and the trafficking of C1q down the secretory pathway was defective. We found that the mature C1q protein was mainly retained in the ER and not transported to the Golgi apparatus to be packaged for secretion.

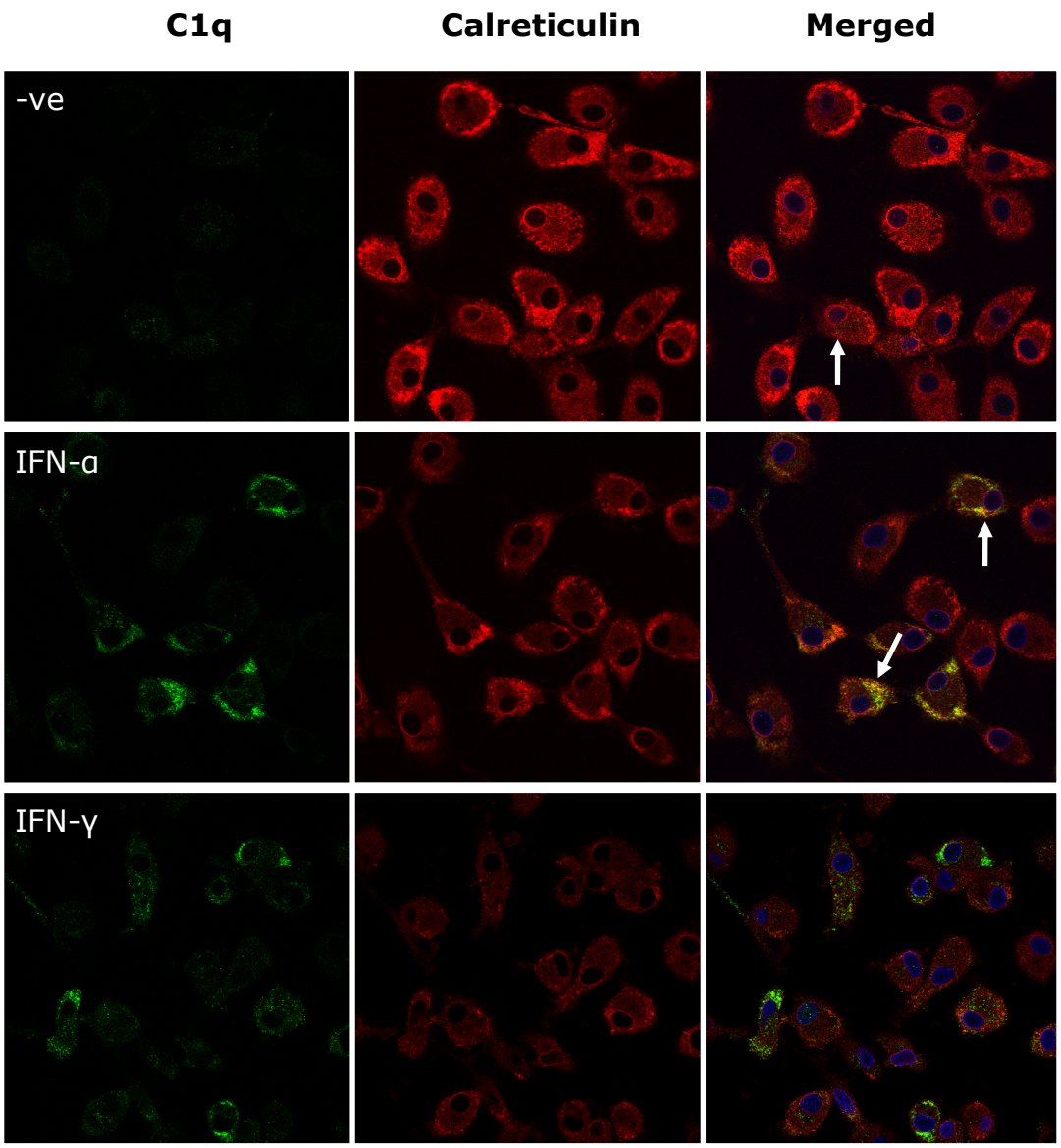

Figure 5.5 C1q is trapped in the ER following IFN-α stimulation for 2 days. Immature DCs (2 X 10^5) differentiated from monocytes for 6 days were seeded onto coverslips laid in wells of a 24-well culture plate. The cells were stimulated IFN-α, IFN-γ or left unstimulated for 2 days. The cells were then washed, fixed and permeabilized with 0.1% saponin. Mouse anti-C1q and rabbit anti-calreticulin antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear as blue signals in the merged image. C1q co-localized with CRT in DCs stimulated with IFN-α, and appear as yellow signals pointed by the arrows. For IFN-γ treated DCs, minimal co-localization of C1q and CRT was observed. C1q in unstimulated cells was not localized in the ER and appeared as distinct green signals indicated by the arrow in the merged image

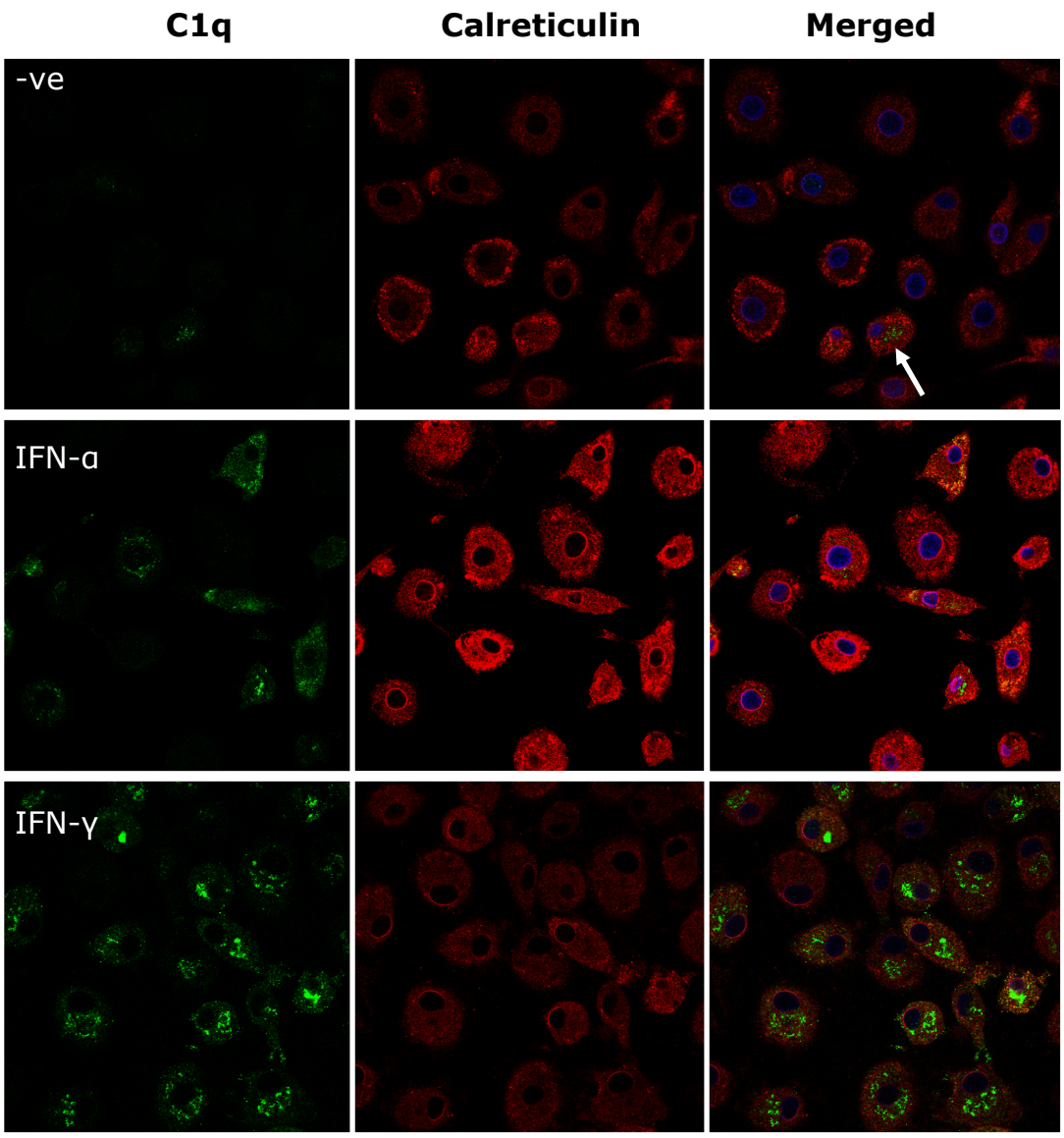

Figure 5.6 C1q is trapped in the ER following IFN-α stimulation for 2 + 2 days. Immature DCs (2 X 10^5) differentiated from monocytes for 6 days were seeded onto coverslips laid in wells of a 24-well culture plate. The cells were stimulated IFN-α, IFN-γ or left unstimulated for 2 days, washed and cultured for another 2 days. The cells were then washed, fixed and permeabilized with 0.1% saponin. Mouse anti-C1q and rabbit anti-calreticulin antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear as blue in the merged image. Most of the C1q co-localized with CRT in DCs stimulated with IFN-α, and appear as yellow signals pointed by the arrows. For IFN-γ treated DCs, C1q and CRT rarely co-localized and appeared as separate signals. C1q in unstimulated cells was not localized in the ER and appeared as distinct green signals indicated by the arrow in the merged image

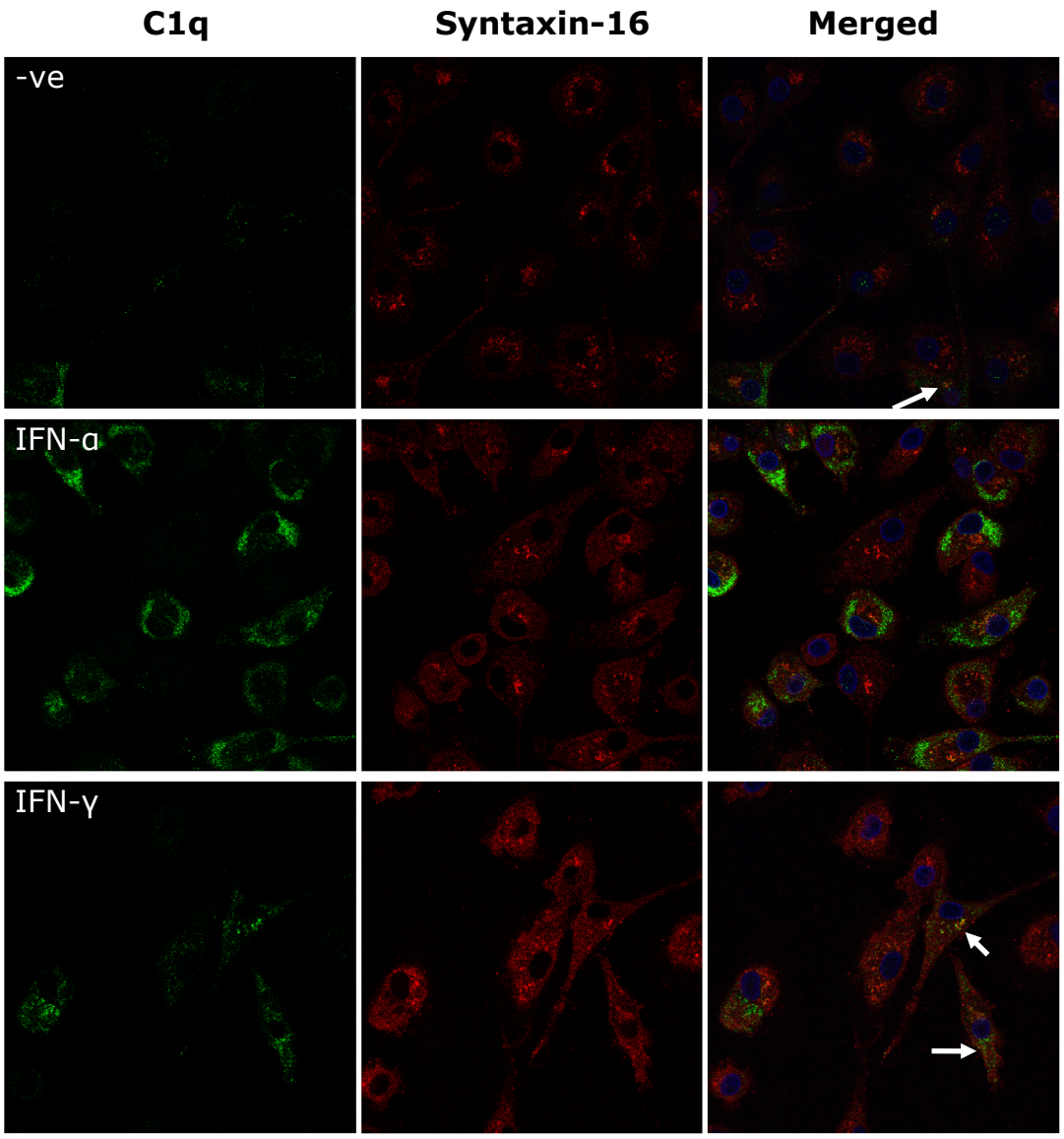

Figure 5.7 Less C1q is transported to the Golgi apparatus for secretion following 2 days of IFN-α stimulation than IFN-γ stimulation. Immature DCs (2 X 10^5) differentiated from monocytes for 6 days were seeded onto coverslips laid in wells of a 24-well culture plate. The cells were stimulated with IFN-α, IFN-γ or left unstimulated for 2 days. The cells were then washed, fixed and permeabilized with 0.1% saponin. Mouse anti-C1q and rabbit anti-Syntaxin-16 antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear blue in the merged image. C1q co-localized with Syn16 in DCs stimulated with IFN-γ, and appears as yellow signals pointed by the arrows. C1q in IFN-α treated DCs appears distinct from Syn16 and the signals are mainly green, showing minimal co-localization with the Golgi apparatus. Some C1q was localized in the Golgi apparatus in the untreated DCs as indicated by the arrow.

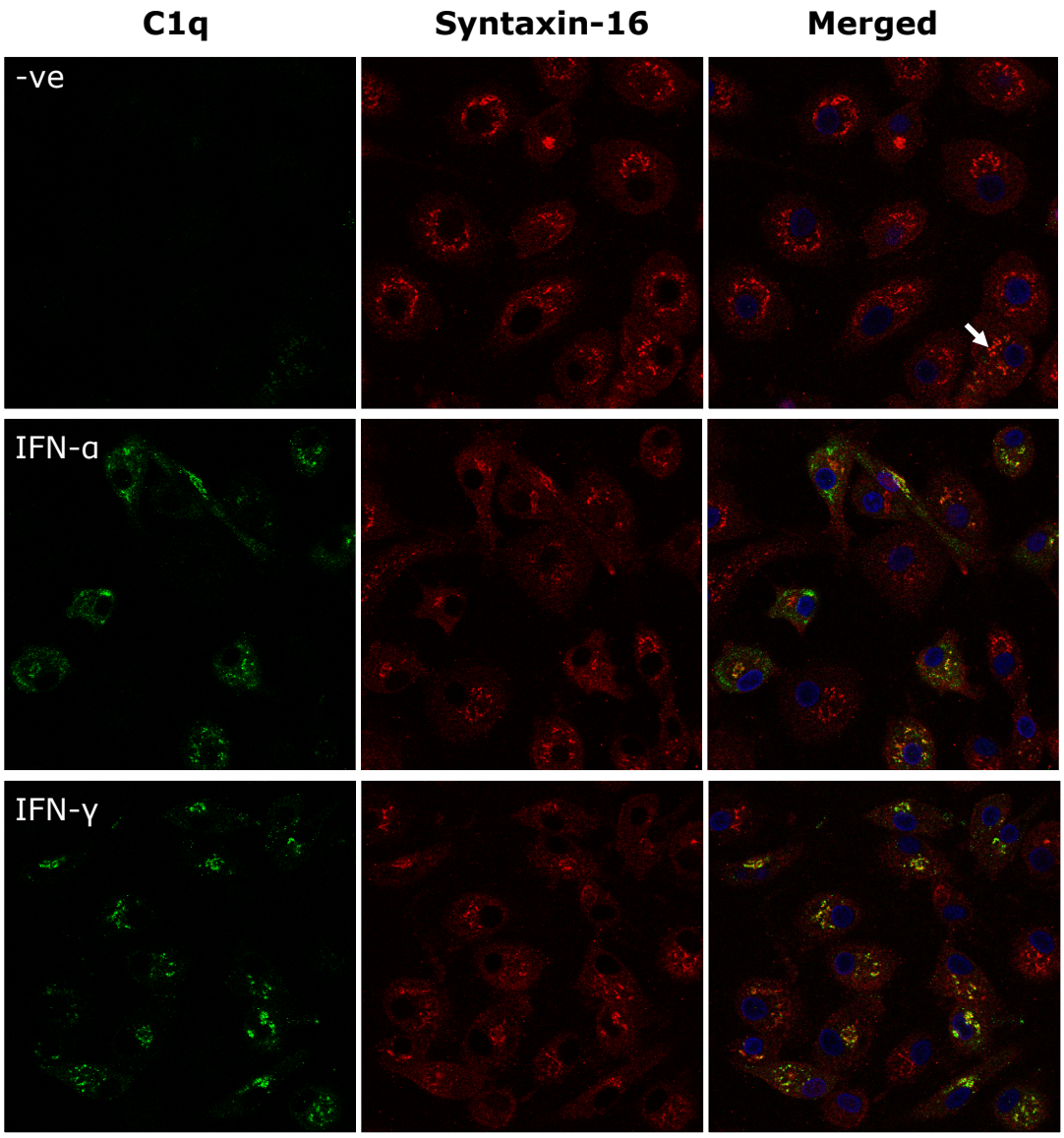

Figure 5.8. Less C1q is transported to the Golgi apparatus for secretion following 2 + 2 days of IFN-α than IFN-γ stimulation. Immature DCs were seeded onto coverslips and stimulated with IFN-α, IFN-γ or left unstimulated for 2 days, washed and cultured for another 2 days. The cells were then washed, fixed and permeabilized. Mouse anti-C1q and rabbit anti-Syntaxin-16 antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear blue in the merged image. Most of the C1q co-localized with Syn16 in DCs stimulated with IFN-γ, and appears as yellow signals. More C1q in 2 + 2 days IFN-α treated DCs localized with Syn16 than the 2 days treated DCs, but is still less compared to IFN-γ stimulated DCs, as most of the C1q still appear as distinct green signals in the merged image (white arrows). Some C1q was localized in the Golgi apparatus in the untreated DCs as indicated by the arrow.

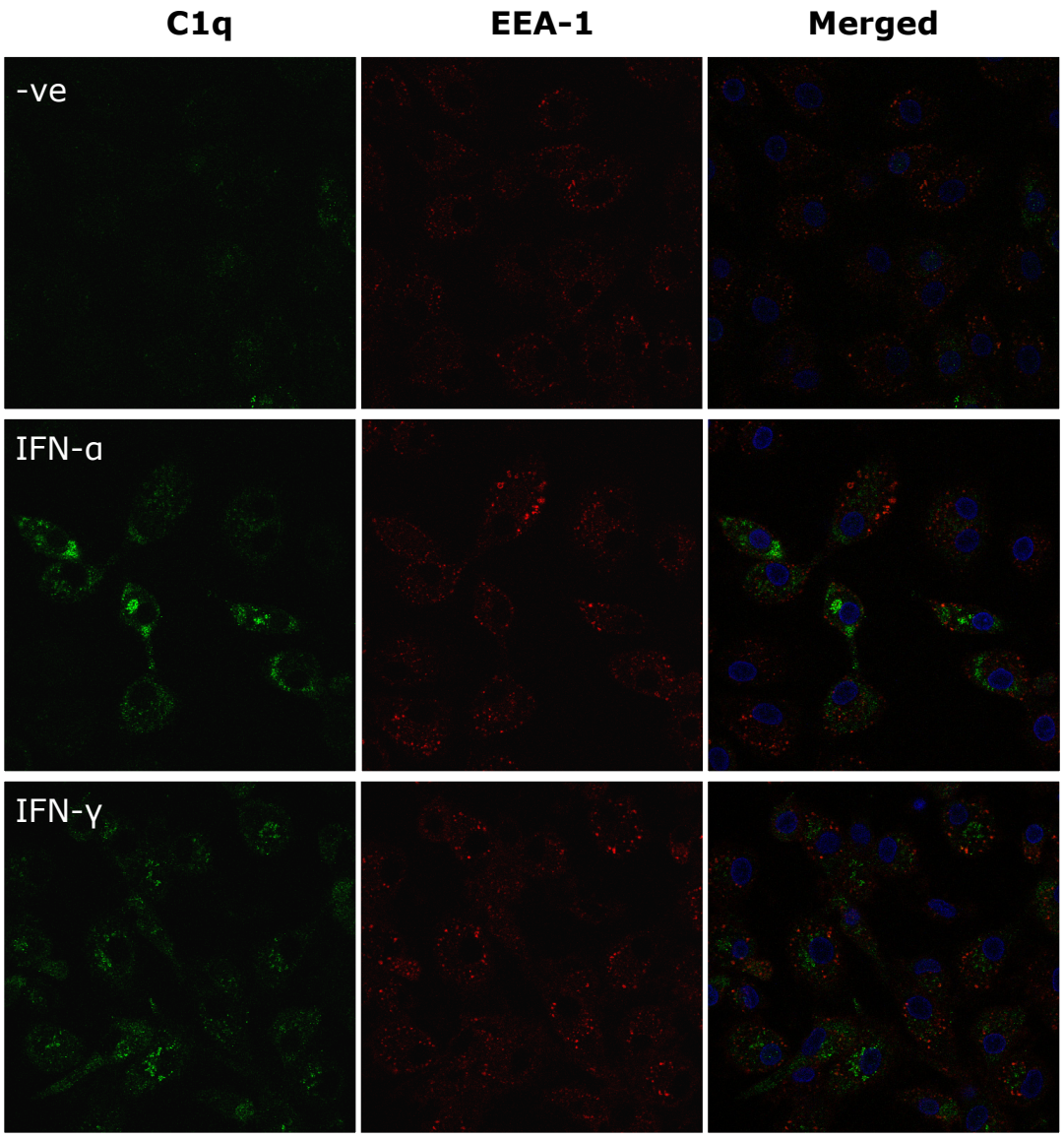

Figure 5.9 C1q is not localized in the early endosome after 2 days culture. Immature DCs (2 X 10^5) differentiated from monocytes for 6 days were seeded onto coverslips laid in wells of a 24-well culture plate. The cells were stimulated IFN-α, IFN-γ or left unstimulated for 2 days. The cells were then washed, fixed and permeabilized with 0.1% saponin. Mouse anti-C1q and rabbit anti-EEA-1 antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear blue in the merged image. C1q did not co-localize with EEA-1 in DCs for all 3 conditions and is thus not located in the early endosomes compartment.

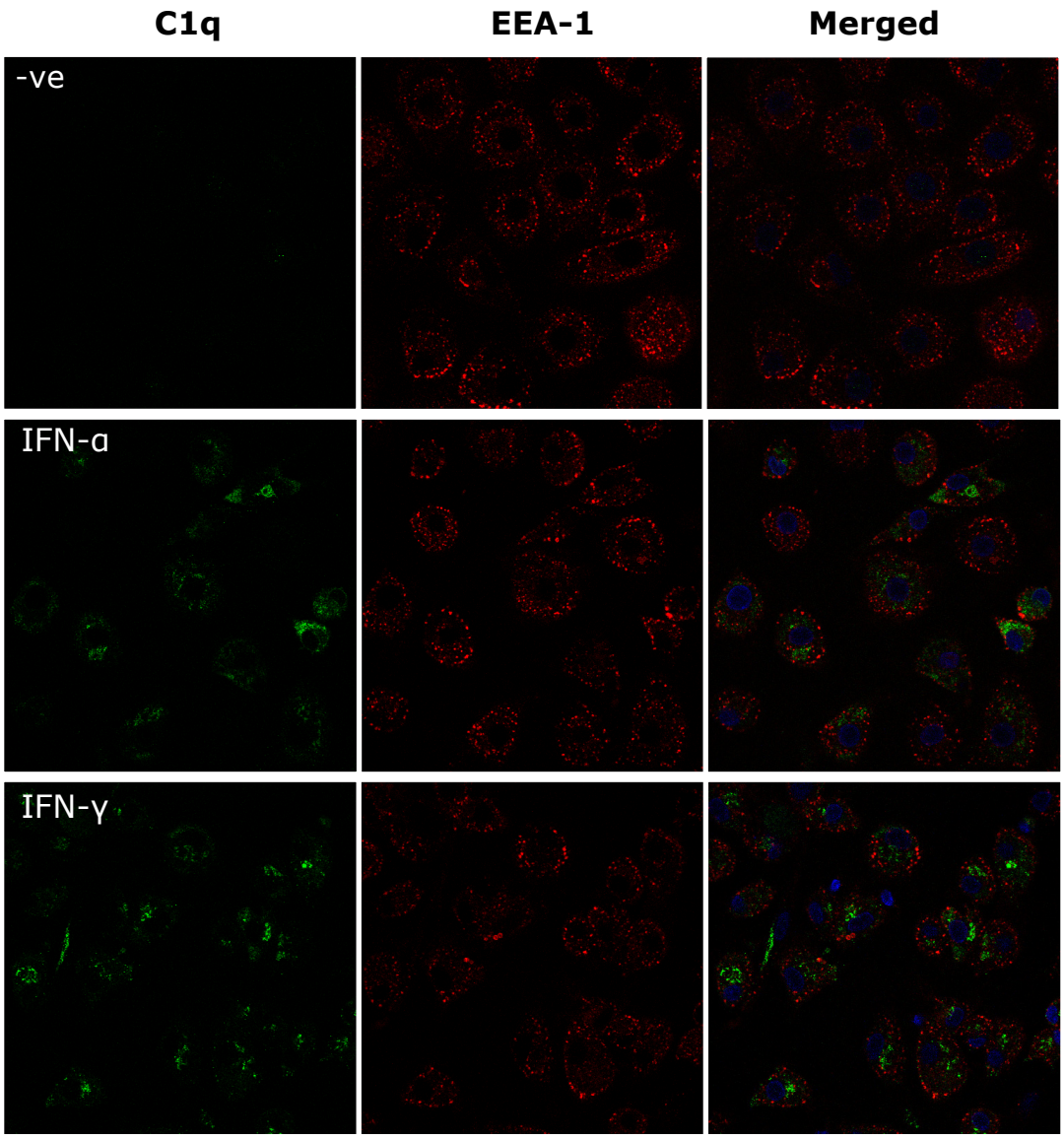

Figure 5.10 C1q is not localized in the early endosome after 2 + 2 days culture. Immature DCs (2 X 10^5) differentiated from monocytes for 6 days were seeded onto coverslips laid in wells of a 24-well culture plate. The cells were stimulated IFN-α, IFN-γ or left unstimulated for 2 days, washed and cultured for another 2 days. The cells were then washed, fixed and permeabilized with 0.1% saponin. Mouse anti-C1q and rabbit anti-EEA-1 antibodies were added and incubated for 1 hr on ice, washed and stained with secondary goat anti-mouse (FITC, green) and goat anti-rabbit (Cy3, red). Nuclei stained with DAPI appear blue in the merged image. C1q did not co-localize with EEA-1 in DCs for all 3 conditions and is thus not located in the early endosomes compartment.

5.8 Fibronectin secretion is not reduced following IFN-α stimulation

We have delineated that DCs subjected to chronic IFN-α stimulation seemed to reduce C1q secretion but not its expression. Whether this is specific to C1q or applies to other proteins remains a question. We chose to investigate the expression of fibronectin following IFN-α and IFN-γ stimulation. Fibronectin (Fn) is expressed by DCs and is found in high levels in the plasma and is an important component of the extracellular matrix (ECM) (Lehtonen *et al.*, 2007).

First, we analyzed Fn mRNA expression in DCs following IFN-α/IFN-γ stimulation as before. After 2 days of IFN-α stimulation, Fn mRNA was unaffected whereas it was halved for IFN-γ stimulation (Fig. 5.11A). When we studied the secretion of Fn after 2 days, IFN-α stimulated DCs secreted about 3 times more Fn than DCs stimulated with IFN-γ (Fig. 5.11B). This roughly corresponds to their respective mRNA expression levels.

For the 2 + 2 days treatment, IFN-γ stimulated DCs secreted 3.5-fold less Fn than DCs receiving IFN-α stimulation (Fig. 5.11B). Considering that the Fn mRNA expression for 2 + 2 days IFN-α and IFN-γ stimulation are about equal (Fig. 5.11A), we can infer that protein secretion was generally not affected by IFN-α treatment. In fact, IFN-γ treatment seems to reduce protein secretion. The inhibition on C1q secretion following prolonged IFN-α may be limited to C1q or an unknown subset of proteins that include C1q.

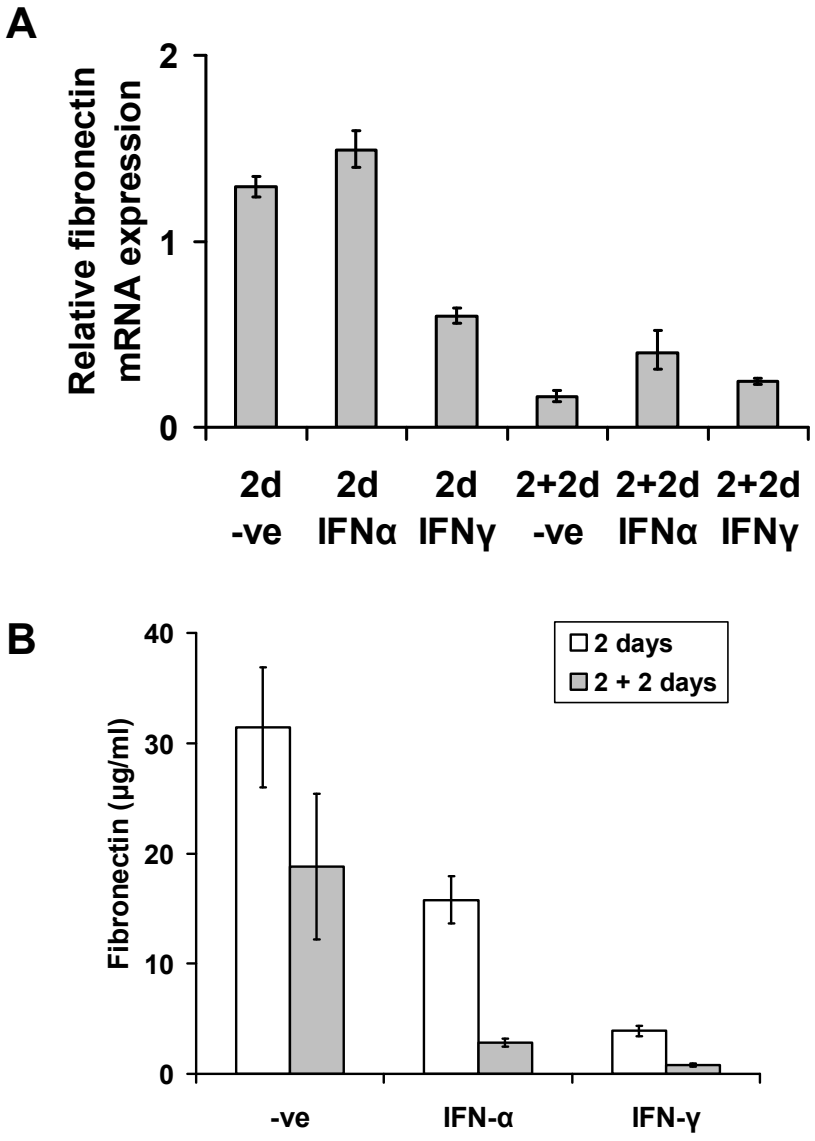

Figure 5.11. Analysis of fibronectin secretion following IFN-α/IFN-γ stimulation. The expression and secretion of the ECM protein fibronectin in DCs after IFN-α and IFN-γ stimulation was studied. **(A)** mRNA from DCs stimulated with both IFNs for 2 days and 2 + 2 days was analyzed for the expression of the Fibronectin-1 gene. **(B)** Culture supernatants for the corresponding time-points and stimuli were also collected and the fibronectin levels were quantitated using an indirect ELISA assay with purified human fibronectin as standards. Stimulations were performed in triplicates and the results are presented as means ± SD.

Chapter 6 Deposited C1q induces differentiation of DCs with tolerogenic properties

6.1 Introduction

After the discovery of tissue deposited C1q around DC in tissues, the function of this C1q fraction has not drawn significant investigations (Cao *et al.*, 2003). In one recent study, it was shown that deposited C1q could directly activate monocyte-derived DCs like LPS but the underlying mechanisms were not investigated (Csomor *et al.*, 2007). In contrast, another study suggested a tolerogenic role for C1q, in that LPS induced less IL-12p40 when excess C1q was added to bone marrow-derived DCs suggesting C1q inhibition of IL-12 production (Yamada *et al.*, 2004).

Another mechanism by which deposited C1q may regulate DCs is to modulate monocyte differentiation into DCs with altered properties. This will be the focus of this section in which we present results showing that DCs cultured on deposited C1q were significantly different from DCs cultured without C1q. Overall, these C1q-differentiated DCs (C1qDCs) were less inflammatory and less able to stimulate Th1 and Th17 activation.

6.2 C1qDCs express the characteristic surface MHC, co-stimulatory, CD83 and CCR7 molecules like normal DCs

To examine whether surface-deposited C1q regulates DC development, monocytes were differentiated to DCs on C1q-coated plates (C1qDCs) or normal plates (normal DCs). Cells were cultured using the same procedure as established in Chapter 3.2, with monocytes seeded at $1.5x10^6$/ml in a total volume of 2 ml and supplemented with IL-4 and GM-CSF. At day 6, the yields of the two DC types were similar (about $0.8x10^6$/ml). As shown in Fig. 6.1, C1qDCs were minimally different from normal DCs in the expression of surface CD1a, CD14, MHC, CD80, CD86 and CD40. Upon activation with the powerful maturation stimuli of LPS + IFN-γ for 48 hr, MHC and co-stimulatory molecules were similarly upregulated and CD83 and CCR7 were similarly induced on the two DC types. Therefore, these C1qDCs have the characteristic normal DC phenotype.

6.3 C1qDCs are less adhesive to cell culture wells than normal DCs

C1qDCs visually showed marked differences from normal DCs. In live cell culture following 6 days of monocyte differentiation, normal DCs form cell clusters and a major fraction of these cells were adherent and spreading (Fig. 6.2A). C1qDCs also formed clusters, but these cells were mostly non-adherent. DCs cultured on BSA-coated plates (BSA-DCs) appeared just as adherant as normal DCs (Fig. 6.2A).

Staining of DCs during the differentiation period with crystal violet also detected less adherent cells in the C1qDC culture (Fig. 6.2B). Since C1q is a highly

positively charged protein (pI = 9.0), we also coated plates with poly-L-lysine to culture DCs. Poly-L-lysine increased rather than decreased DC adhesion (Fig. 6.2B). It shows that the reduced adhesion by C1qDCs was not due to the positively charged nature of the coated C1q and is a characteristic specific to the C1qDCs.

6.4 C1qDCs phagocytose more apoptotic cells than normal DCs

Failed clearance of apoptotic cells (AC) is postulated to be a mechanism that may lead to SLE pathogenesis (Truedsson *et al.*, 2007). To examine whether C1qDCs are different from normal DCs in AC phagocytosis, CFSE-labeled ACs were prepared from Jurkat cells by UV-irradiation and added to DCs at a 1:1 ratio. After AC incubation with DCs, extracellular CFSE fluorescence was quenched with trypan blue. The cells were then stained for CD11c to identify DCs. In this experiment, CFSE signals detected in $CD11c^+$ cells portrays DCs that have phagocytosed AC (Fig. 6.3). When the incubation was performed at 4ºC, minimal AC phagocytosis was detected with either DC type (2.3% and 3.5% respectively for C1qDCs and normal DCs) as there was no cytoskeletal rearrangement and actin polymerization. Incubation at 37ºC resulted in 31% phagocytosis with normal DCs but a significantly higher 49% with C1qDCs (Fig. 6.3). Therefore, C1qDCs are likely to be more potent than normal DCs in AC clearance.

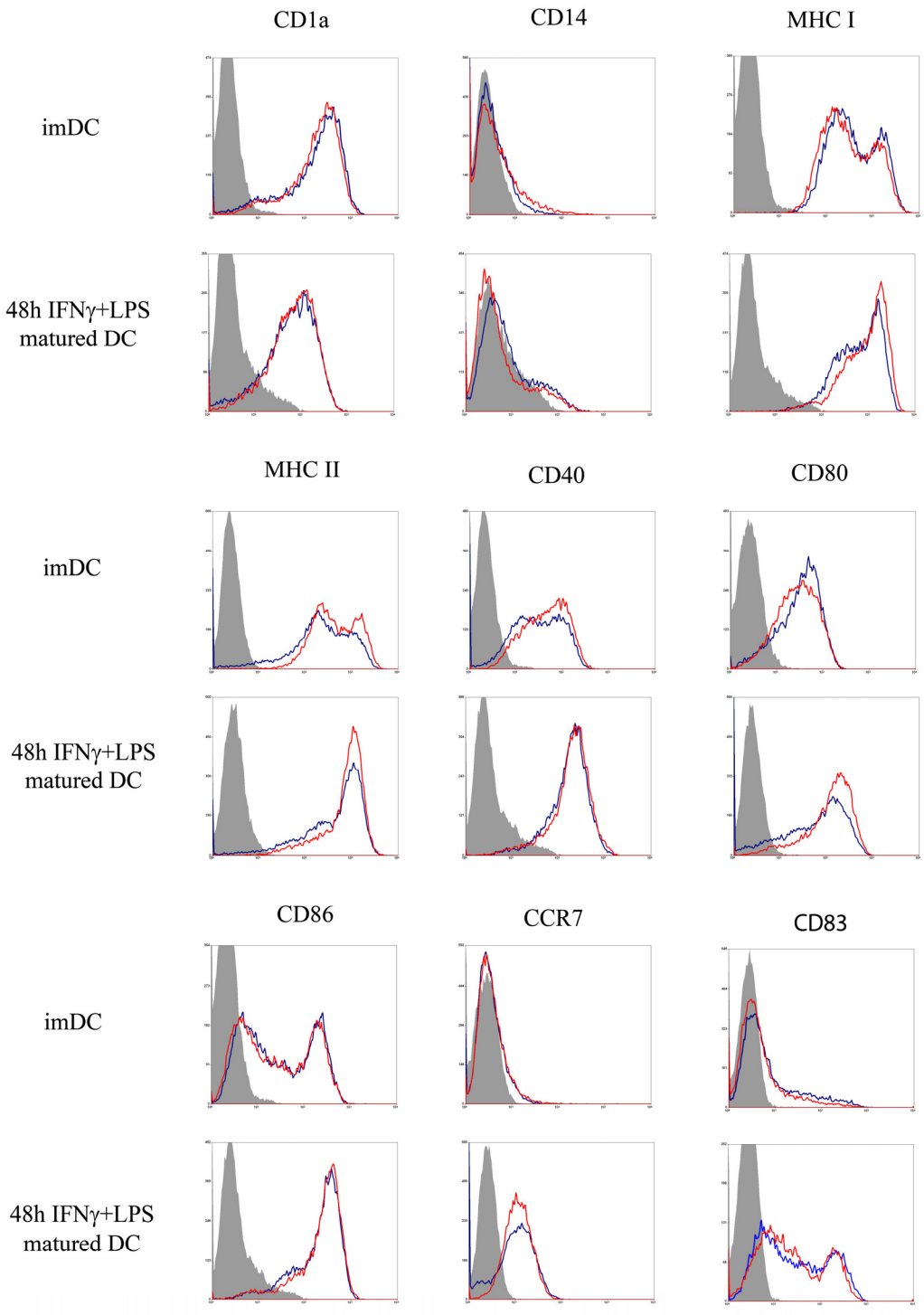

Figure 6.1. Phenotype of C1qDCs and normal DCs. C1qDCs and normal DCs were cultured from monocytes and, at day 6 (imDCs), the cells were stained with fluorochrome-conjugated specific antibodies. Some imDCs were stimulated with LPS/IFN-γ for 2 days (matured DC) before staining with the same antibodies. Solid histograms, isotype IgG; red histograms, C1qDCs; blue histograms, normal DCs.

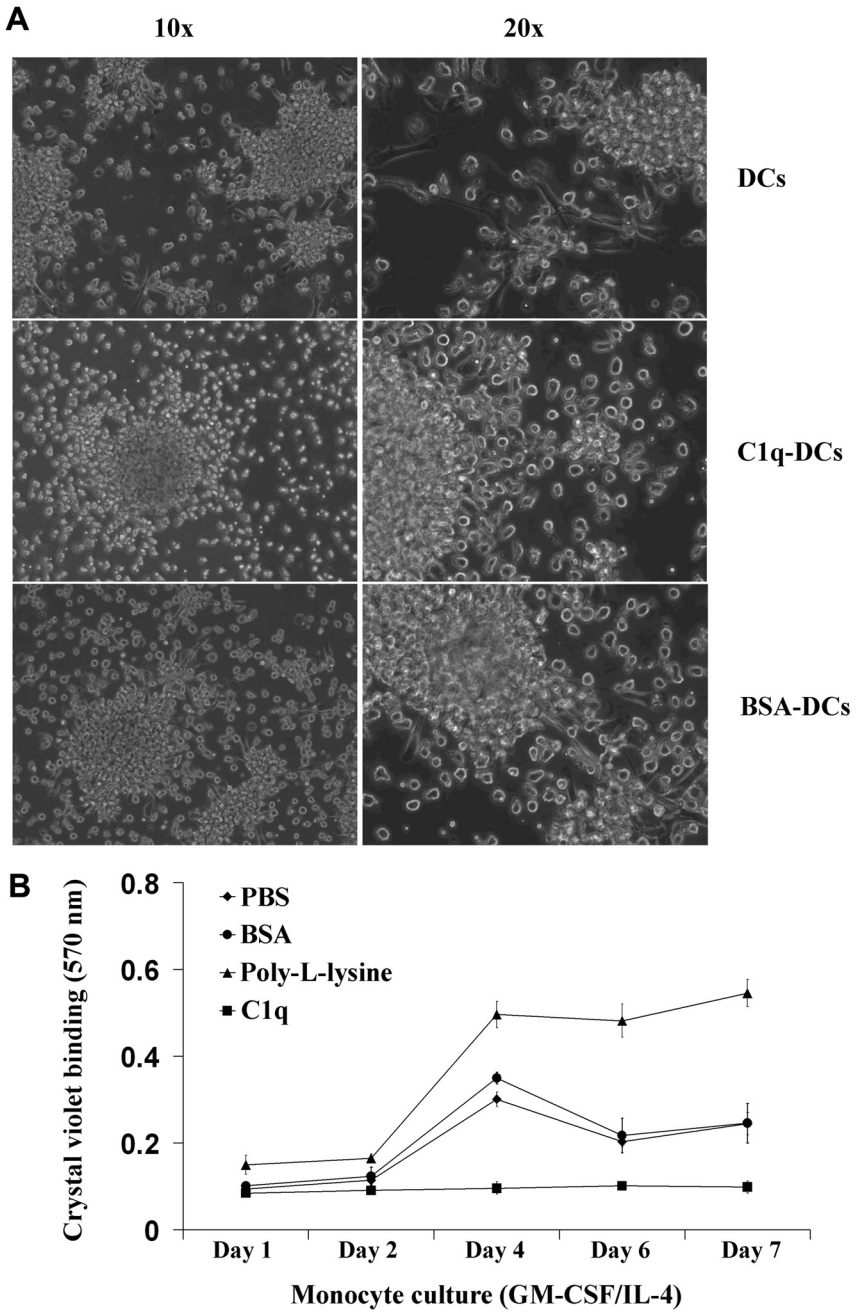

Figure 6.2. Adhesion of C1qDCs and normal DCs. (A) Cultures of normal DCs, C1qDCs and BSA-DCs were examined at day 6. Images were captured using 10x and 20x objectives. Note the presence of adherent and spreading cells for normal and BSA-DCs when viewed under the 20x objective. **(B)** Monocytes were cultured in 96-well plates ($2x10^5$/well) to generate DCs. At the indicated time-points, non-adherent cells were removed by washing and adherent cells were stained with crystal violet. Cell-bound dye was eluted and measured at 570 nm. In this experiment, plates were coated with C1q, BSA or poly-L-lysine (all 50 μg/ml) or PBS as a control. These experiments were performed in triplicates and results are presented as means ± SD.

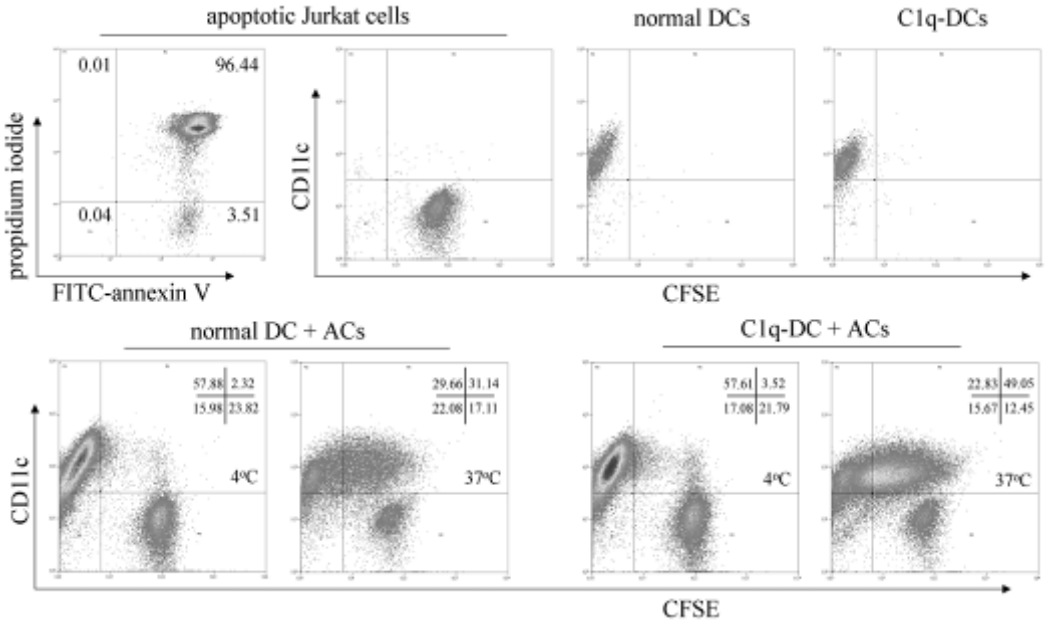

Figure 6.3. C1qDCs display enhanced phagocytosis of AC. Jurkat cells were labeled with CFSE, UV-irradiated and cultured for 4 hr in serum-free RPMI1640 to induce apoptosis. This was assessed by propidium iodide and Annexin V staining. In the phagocytosis experiments, apoptotic Jurkat cells were incubated with normal DCs or C1qDCs in RPMI 1640 + 10% human serum for 2 hr at 37^{o}C. In each experiment, 1x10^{5} DCs were incubated with 1x10^{5} AC in 0.2 ml volume. As a control, the cells were incubated at 4^{o}C. Excessive fluorescence from non-engulfed cells was quenched in cold Trypan blue, washed and the cells were fixed with 4% PFA. DCs were identified based on CD11c (PE) staining in flow cytometry.

6.5 C1qDCs produce less inflammatory cytokines TNF-α, IL-6 and IL-12 and IL-23 but more anti-inflammatory cytokine IL-10 than normal DCs

With respect to IL-8, IL-6 and TNF-α production, the two DC types produced similar levels of IL-8 after LPS or LPS/IFN-γ stimulation but C1qDCs produced significantly less IL-6 and TNF-α than normal DCs (Fig. 6.4). With LPS stimulation, BSA-DCs also produced less IL-6 and TNF-α but this was not observed with LPS/IFN-γ co-stimulation. Overall, C1qDCs produced less inflammatory cytokines.

We then examined IL-10, IL-12 and IL-23 production, which are intimately associated with the ability of DCs to stimulate adaptive immunity or autoimmunity (Oppmann *et al.*, 2000). With LPS stimulation, C1qDCs consistently produce more IL-10 but less IL-12 (Fig. 6.4). Bioactive IL-12 (IL-12p70), which is a p35/p40 heterodimer, was not induced with LPS alone from both DC types. IFN-γ co-stimulation is required for IL-12p70 induction (Liu *et al.*, 2003). LPS only induced IL-12p40, but much less IL-12p40 was induced from C1qDCs as compared with normal DCs. With LPS/IFN-γ co-stimulation, IL-12p70 was induced but C1qDCs also produced less IL-12p70 than normal DCs. As a control, BSA-DCs were similar to normal DCs in IL-10 and IL-12 production.

Besides IL-12p35, IL-12p40 also dimerizes with IL-23p19 which forms bioactive IL-23, a cytokine which stimulates Th17 cell survival and effector cytokine production (Oppmann *et al.*, 2000). LPS induced half the levels of IL-23 from C1qDCs as compared with normal DCs. LPS/IFN-γ co-stimulation induced IL-23 more strongly, but C1qDCs still produced half the amount of IL-23 as normal DCs.

Therefore, DCs cultured on surface-deposited C1q, mimicking tissue-deposited C1q, selectively increase IL-10 but decrease IL-12 and IL-23 production when activated.

Significant donor variations were observed throughout this study. C1qDCs can sometimes produce as up to 5-fold less IL-12p70 than normal DCs and similarly about 5-fold more IL-10. C1qDCs from other donors may not show such a striking difference and sometimes less than 2-fold less IL-12p70 or less than 2-fold more IL-10 could be observed. The absolute levels of cytokines fluctuate between donors, with IL-12p70 levels varying the most out of all cytokines, from less than 100 pg/ml detected up to more than 5 ng/ml. However, it was consistently observed that C1qDCs produced significantly more IL-10 and less IL-12p70 than normal DCs.

6.6 C1qDCs induce less Th1 and Th17 cells than normal DCs

The reduced IL-12 and IL-23 production by C1qDCs implies weaker Th1 and Th17 cell stimulation and this was examined by MLR. Naïve and memory $CD4^+$ T cells that were more than 90% pure (Fig. 6.5) were isolated from PBMC for MLR co-culture with C1qDCs, normal DCs and macrophages cultured with M-CSF as a control. These macrophages produce high IL-10 but little IL-12 and IL-23 (data not shown). After co-culture of normal DCs with T cells for 7 day, a large population of IFN-γ-producing Th1 cells was induced from naïve (25%) and memory (28%) cells. The IL-17-producing Th17 cells were only induced from memory cells (1.1%) (Fig. 6.6). A significant cell population (0.9%) was induced to produce both IFN-γ and IL-17. In contrast, much fewer Th1 and Th17 cells were induced by macrophages.

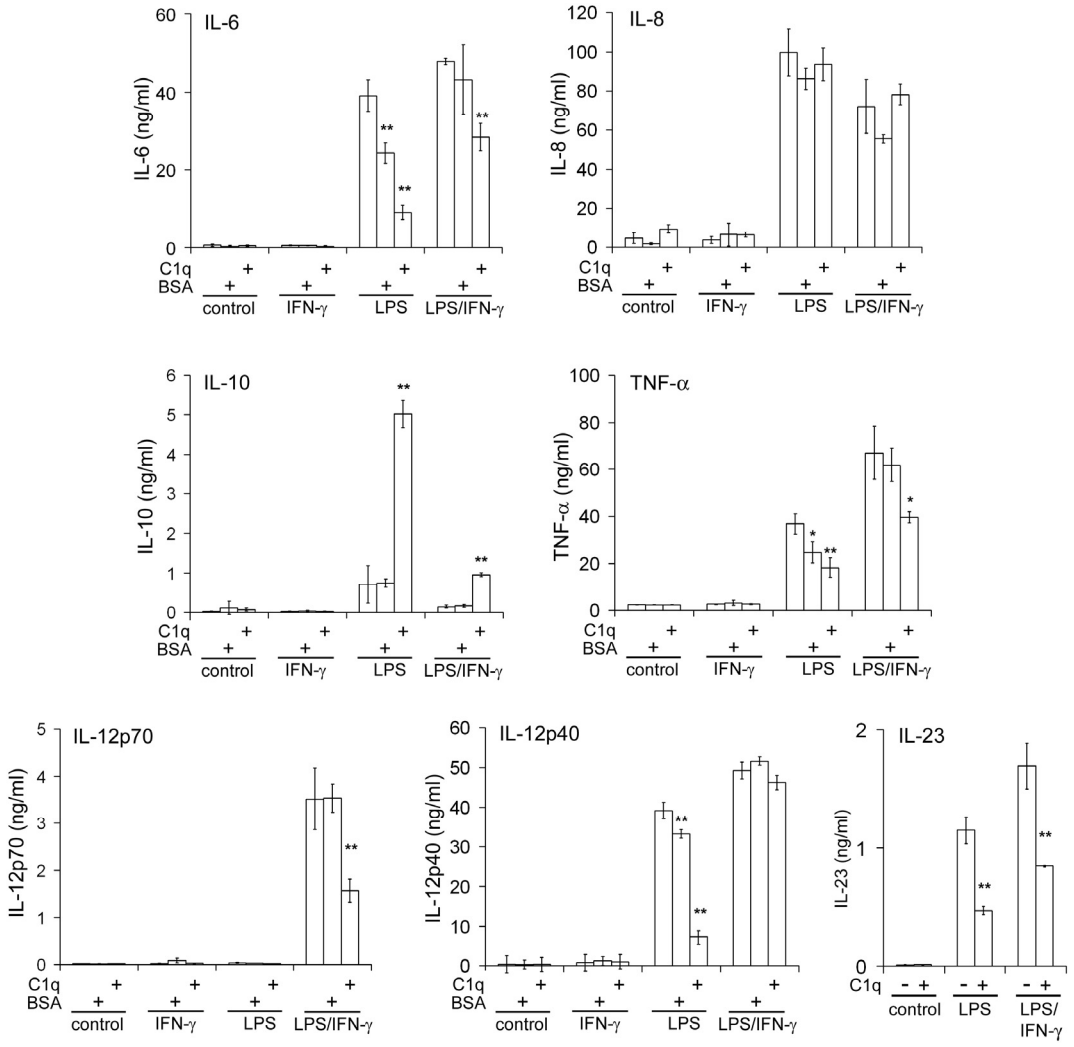

Figure 6.4. Distinctive anti-inflammatory cytokine production profile by C1qDCs. DCs (7 X 10^4 per well) were stimulated for 24 hr in 96-well plates with IFN-γ, LPS, LPS/IFN-γ or left unstimulated (control). Cytokine production was determined by ELISA. As a control, DCs were cultured on BSA-coated plates (BSA-DC). IL-23 was measured in separate experiments. All experiments were performed in triplicates and results are presented as means ± SD.

Compared with normal DCs, C1qDCs induced intermediate levels of Th1 and Th17 cells. C1qDCs induced 9% and 17% IFN-γ positive Th1 cells from naïve and memory cells respectively (Fig. 6.6). From memory cells, C1qDCs induced 0.6%

Th17 cells. The population of T cells that produce both IL-17 and IFN-γ was 0.2% with C1qDCs. Therefore, C1qDCs are clearly weaker than normal DCs in Th1 and Th17 cell induction. This is consistent with their lower IL-12 and IL-23 production than normal DCs (Fig. 6.4).

6.7 C1qDCs induce less IFN-γ and IL-17 secretion from allogeneic $CD4^+$ T cells

IFN-*γ* and IL-17 secretion were also measured in these MLR experiments. In agreement with the results of the intracellular cytokine detection (Fig. 6.6), C1qDCs also induced less IFN-*γ* secretion from the $CD4^+$ T cells (Fig. 6.7A). In these experiments, IFN-*γ* (0.1 μg/ml) was added as a stimulus which was expected to interfere with IFN-*γ* detection by ELISA. We then washed the cells at day 7 and re-stimulated the T cells with anti-CD3 and anti-CD28 antibodies coated onto beads. In these re-stimulation experiments, T cells that were primed with C1qDCs or macrophages in MLR, produced low amounts IFN-*γ* after re-stimulation. In contrast, T cells primed with normal DCs showed potent IFN-*γ* production upon re-stimulation, irrespective of whether naïve or memory cells were used in the initial MLR (Fig. 6.7B). It was also noticed that, with normal DCs in MLR, naïve cells produce twice as much IFN-*γ* as memory cells when these cells were re-stimulated with bead-coated CD3 and CD28 antibodies (Fig. 6.7B).

The polarization of adaptive immunity towards a Th1 phenotype critically requires IL-12 (Murphy *et al.*, 2000). As high levels of IL-12 secretion are observed when

normal DCs were stimulated with IFN-γ + LPS (Fig. 6.4), we sought to clarify if this is the mechanism behind the superior induction of IFN-γ production by normal DCs compared to C1qDCs. We co-cultured total CD4 T cells with both DCs, and IFN-γ + LPS was added. Normal DCs induced about 3-fold more IFN-γ than C1qDCs (Fig. 6.8). When a blocking antibody against IL-12 was added, the IFN-γ production induced by both DCs dropped to almost basal levels (the levels detected in DC alone + IFN-γ/LPS). The IFN-γ levels were not affected when an irrelevant isotype control antibody was used.

In MLR, IL-17 secretion was not detectable with naïve T cells. With memory cells, C1qDCs and macrophages induced low IL-17 secretion (~50 pg/ml), but 5-fold more IL-17 was induced by normal DCs (~250 pg/ml) (Fig. 6.7C). In the subsequent re-stimulation experiments, more IL-17 was detected in T cells that were primed by normal DCs in MLR, produced twice the amount of IL-17 than T cells primed by C1qDCs or macrophages (Fig. 6.7D).

Regulatory T cells (Treg) play an important role in the maintenance of peripheral immunological self-tolerance by suppressing excessive effector T-cell proliferation and activation that may lead to generation of self-reactive T cells (Bonelli *et al.*, 2010). We thus stained the T cells following MLR culture for the Treg markers CD4, CD25 and FoxP3. There appears to be no difference in the ability of both normal DCs and C1qDCs to induce Treg cells following co-culture with naïve CD4 T cells (Fig. 6.9).

Collectively, these results show that C1qDCs are weaker than normal DCs in Th1 and Th17 stimulation. We found that the ability of normal DCs to produce high levels of IL-12 is coupled to its superior ability in inducing IFN-γ producing CD4 T cells compared to C1qDCs. The induction of Treg cells does not seem to contribute to the weaker activation of CD4 cells by C1qDCs.

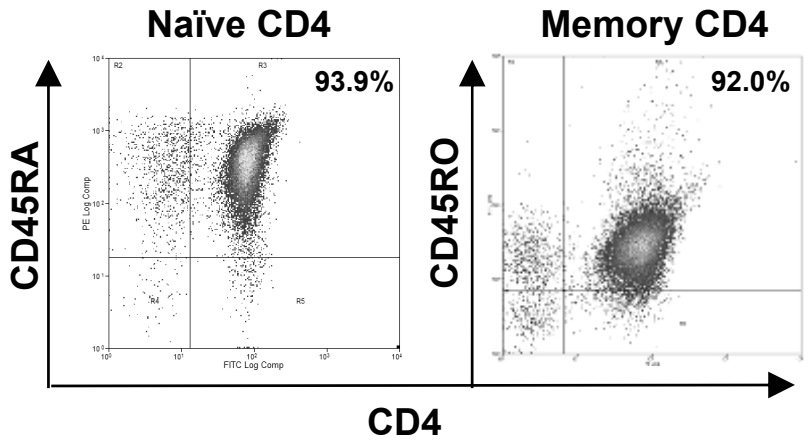

Figure 6.5. Purity of naïve and memory CD4 cells. Both subsets of CD4 T cells were isolated using MACS from PBMCs of healthy donors. The purity was determined by flow cytometry analysis. Naïve cells are $CD4^+$ and $CD45RA^+$ while memory cells are $CD4^+$ and $CD45RO^+$. Cells isolated were always more than 90% pure.

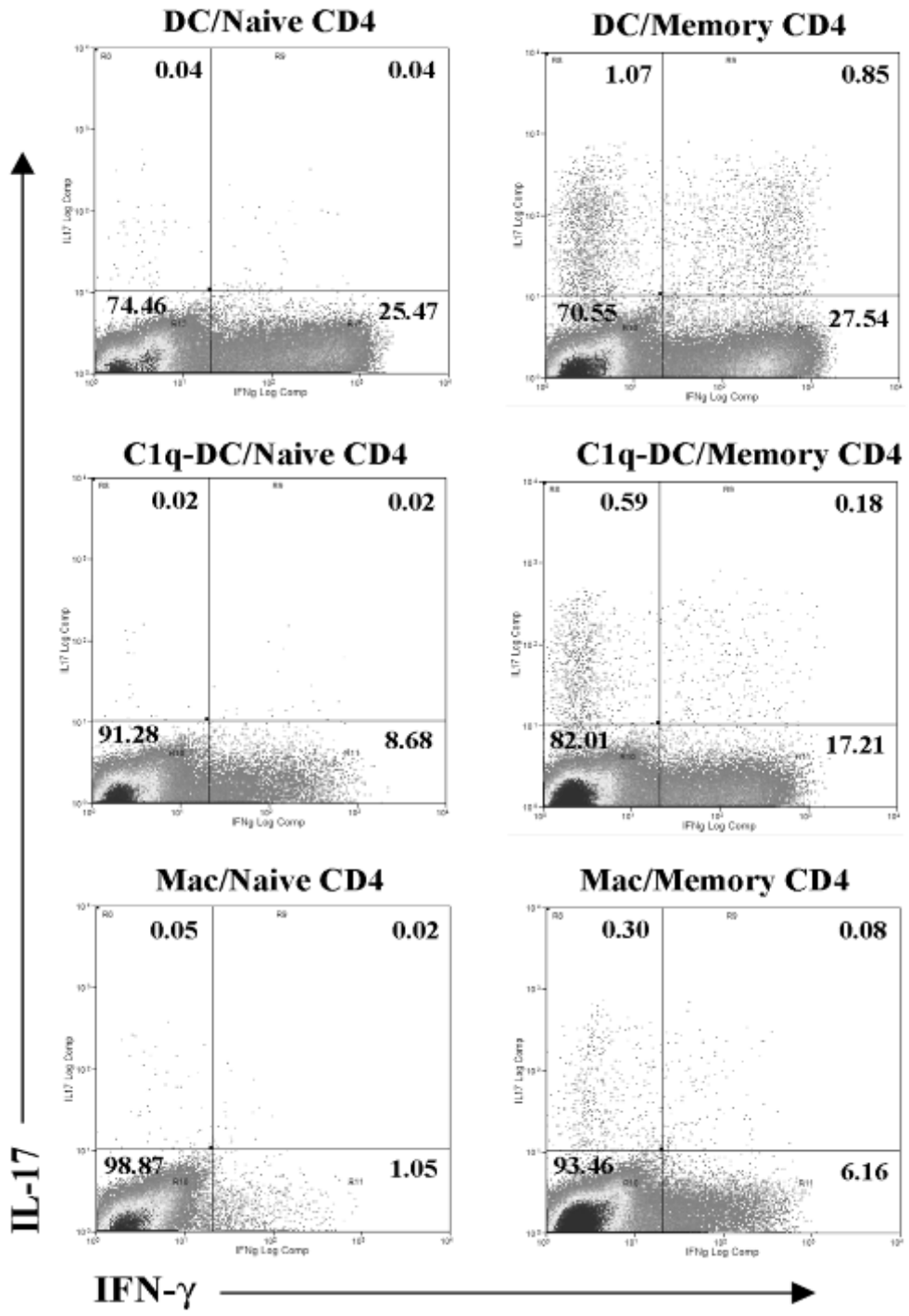

Figure 6.6. Less Th1 and Th17 T cells are induced by C1qDCs than normal DCs. Naïve and memory CD4 T cells were isolated by MACS. In 96-well plates, DCs or macrophages (Mac) ($4x10^4$) were co-cultured with the T cells ($4x10^5$) (0.2 ml/well). LPS and IFN-γ were added to all cultures. At day 7, cells were treated with PMA and ionomycin for a further 6 hr and Brefeldin A was added for the last 4 hr. The cells were fixed, permeabilized, and stained with anti-IL-17 (AlexaFluor-647) and anti-IFN-γ (PE) antibodies before analysis.

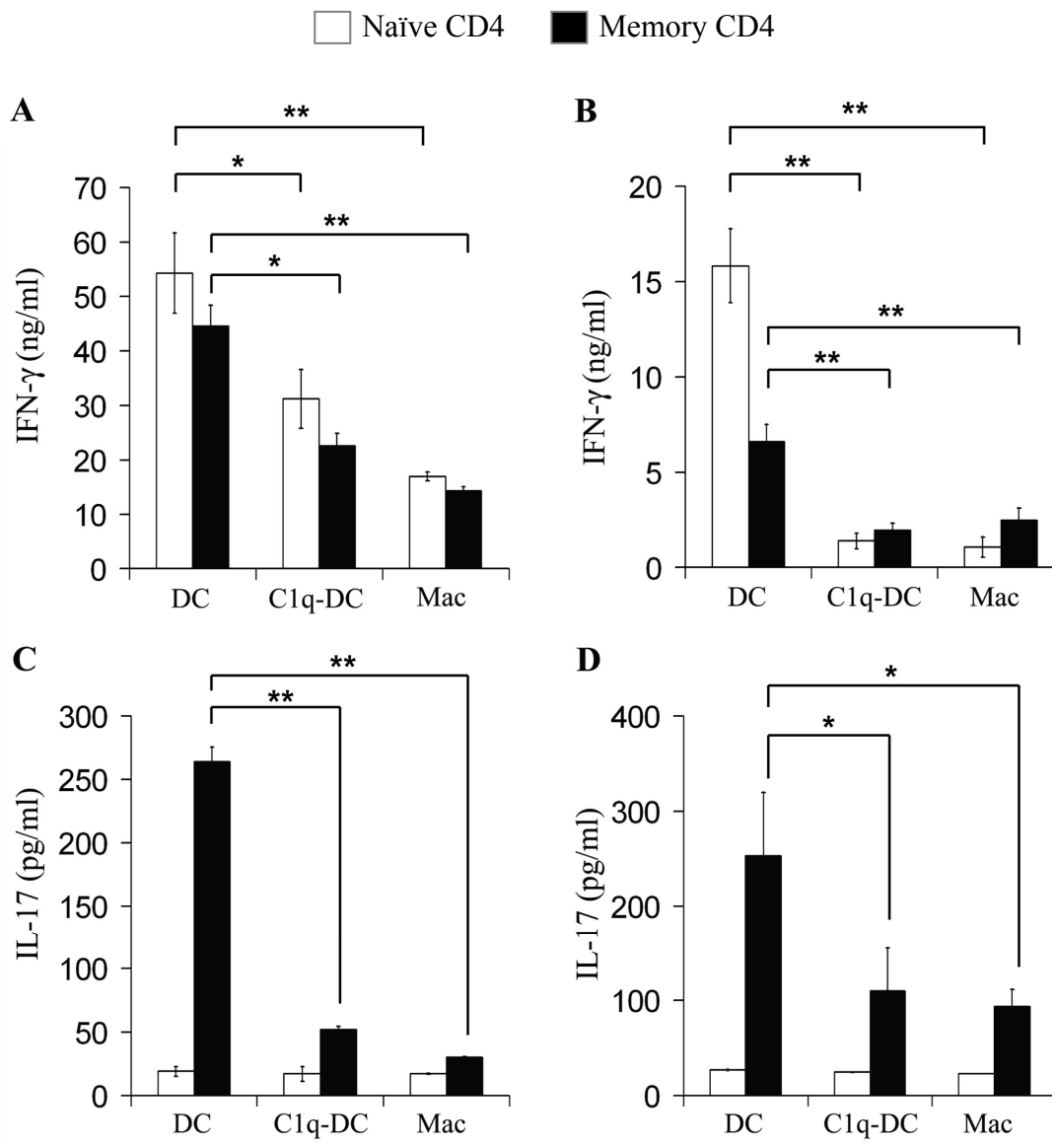

Figure 6.7. Induction of IFN-γ and IL-17 secretion from CD4 T cells by C1qDCs and normal DCs. Naïve (white bar) and memory (black bar) CD4 T cells were isolated and co-cultured with both normal and C1q DCs or macrophages (Mac). (A&C) $4x10^4$ DCs or macrophages were co-cultured with $2x10^5$ naïve or memory CD4 T cells. After 7 days, IFN-γ and IL-17 were determined by ELISA. (B&D) At day 7, cells were washed and re-stimulated with bead-bound CD3 and CD28 antibodies in 96-well plates at bead:cell ratio of 2:1. After 2 days, IFN-γ and IL-17 secretion was measured. Experiments were performed in triplicates and results are presented as means ± SD.

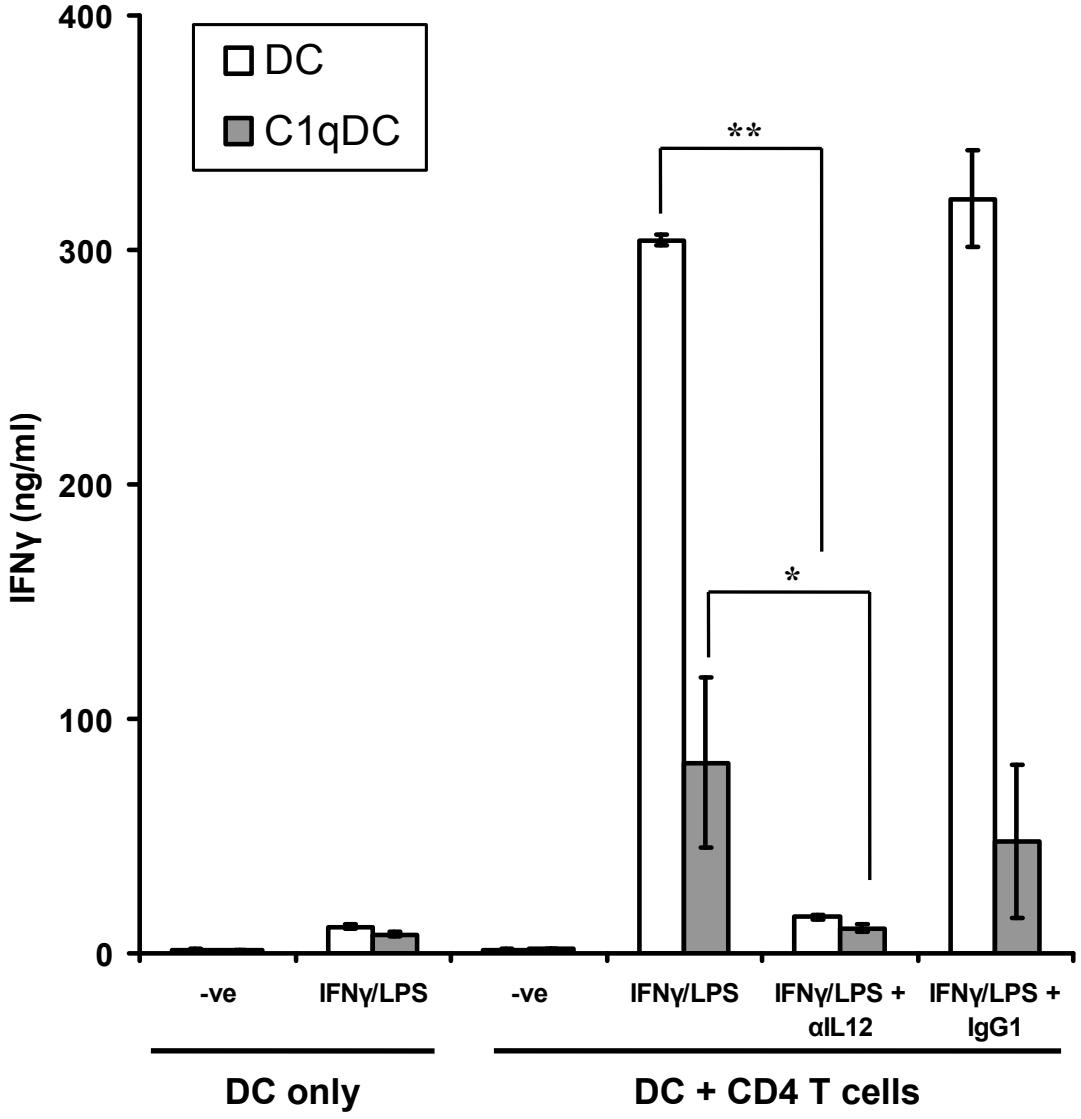

Figure 6.8. The superior induction of CD4 T cell IFN-γ production by normal DCs is coupled to its IL-12 production. Total CD4 T cells were isolated and co-cultured with DCs or C1qDCs. IFN-γ/LPS were added to some cells to induce a strong Th1 signature on the T cells. A blocking anti-IL-12 antibody or the isotype IgG1 control was added (10 μg/ml) to some of the cells. At day 7, culture supernatant was collected and IFN-γ levels were measured. Experiments were performed in triplicates and results are presented as means ± SD.

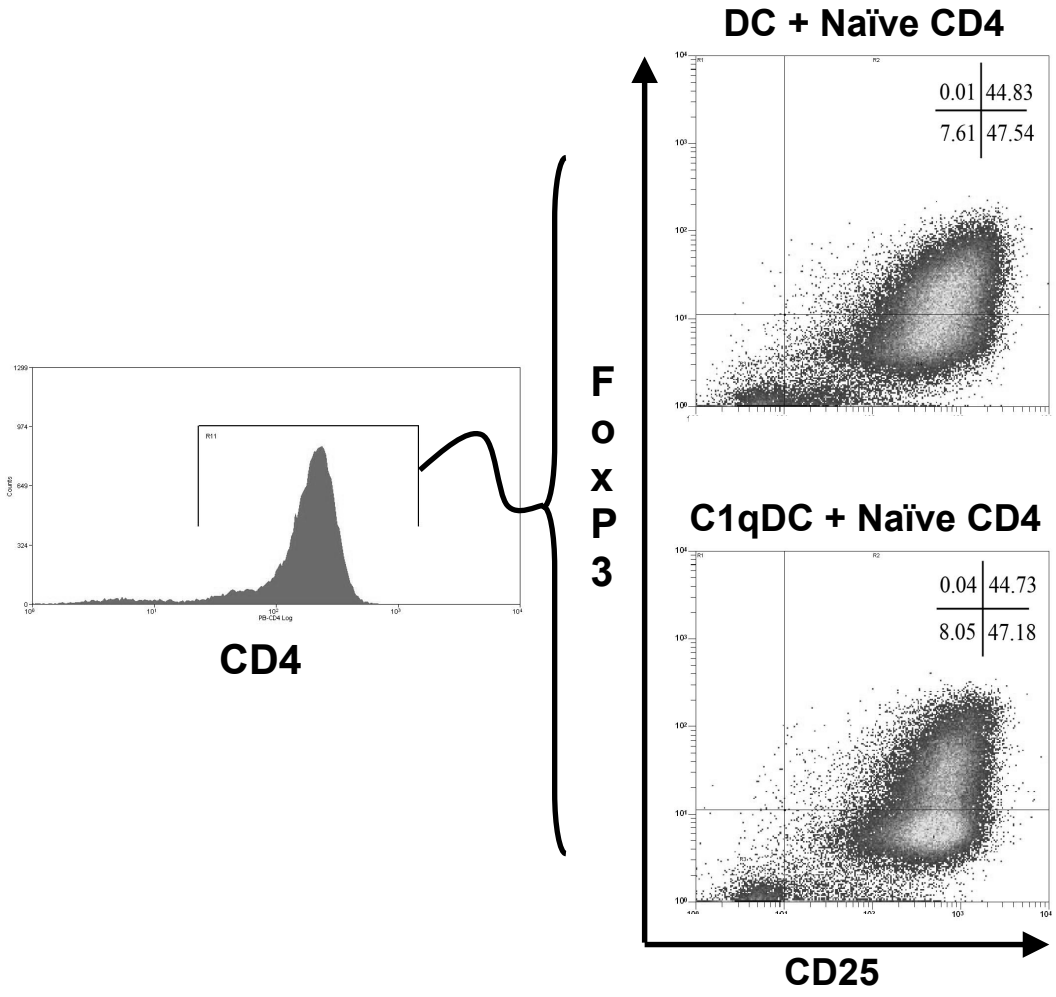

Figure 6.9. No significant difference in the induction of regulatory T cells (Treg) was observed between C1qDCs and normal DCs. Naïve CD4 T cells were isolated and co-cultured with DCs in 96-well plates ($2x10^5$ T cells to $4x10^4$ DCs) At day 7, cells were washed and re-stimulated with beads coated with CD3 and CD28 antibodies at a beads:T cell ratio of 2:1. After 3 days, the cells were harvested and stained for surface CD4 (Pacific blue-conjugated antibody) and CD25 (Alexa647-conjugated antibody). The cells were then permeabilized and stained with a PE-conjugated anti-FoxP3 antibody. Dead cells and debris were excluded by forward and side scatter, and the Treg cells were gated based on the CD4 population and analyzed for CD25 and FoxP3 expression. Results are representative of 3 independent experiments.

6.8 Maturation stimuli attenuate C1qDCs, but enhance normal DCs, in activating naïve T cell

In view of the similar MHC and co-stimulatory molecule profiles on C1qDCs and normal DCs (Fig. 6.1), we asked whether they similarly activate naïve $CD4^+$ T cells by measuring naïve T cell proliferation and surface CD25 induction. As shown in Fig. 6.10, immature C1qDCs and normal DCs were similarly potent in stimulating T cell proliferation. When LPS was added, it caused reduced T proliferation in both C1qDCs or normal DCs. The presence of both LPS and IFN-γ further reduced T cell proliferation caused by both DC types. Nonetheless, no significant difference was observed between C1qDCs and normal DCs in stimulating naïve T cell proliferation.

With respect to CD25 induction on naïve T cells, immature C1qDCs were as potent as immature normal DCs (MFI = 58 and 60, respectively) (Fig. 6.11). The presence of LPS increased CD25 induction by normal DCs (MFI = 84) but it attenuated CD25 induction by C1qDCs (MFI = 36). When both LPS and IFN-γ were present, CD25 induction by normal DCs was further increased (MFI = 156), but its induction by C1qDCs remained low (MFI = 40).

Similar conclusions can be reached from the re-stimulation experiments. Naïve $CD4^+$ T cells primed by immature C1qDCs and normal DCs both expressed high surface CD25 in the re-stimulation experiments (MFI = 332 and 242, respectively) (Fig. 6.11, lower panel). In fact, C1qDCs appeared to be more potent than normal DCs in CD25 induction in their immature states. However, when naïve T cells were stimulated with C1qDCs or normal DCs in the presence of LPS or LPS/IFN-γ, those T cells primed with normal DCs continue to express high CD25 in the re-

stimulation experiments. However, those T cells primed with C1qDCs showed markedly attenuated CD25 expression upon re-stimulation with MFI values dropped to 160 and 59 respectively from 332.

Therefore, maturation stimuli disparately regulate naïve T cell activation depending on whether C1qDCs or normal DCs are used. Normal DCs became more potent after activation but C1qDCs became less potent upon activation.

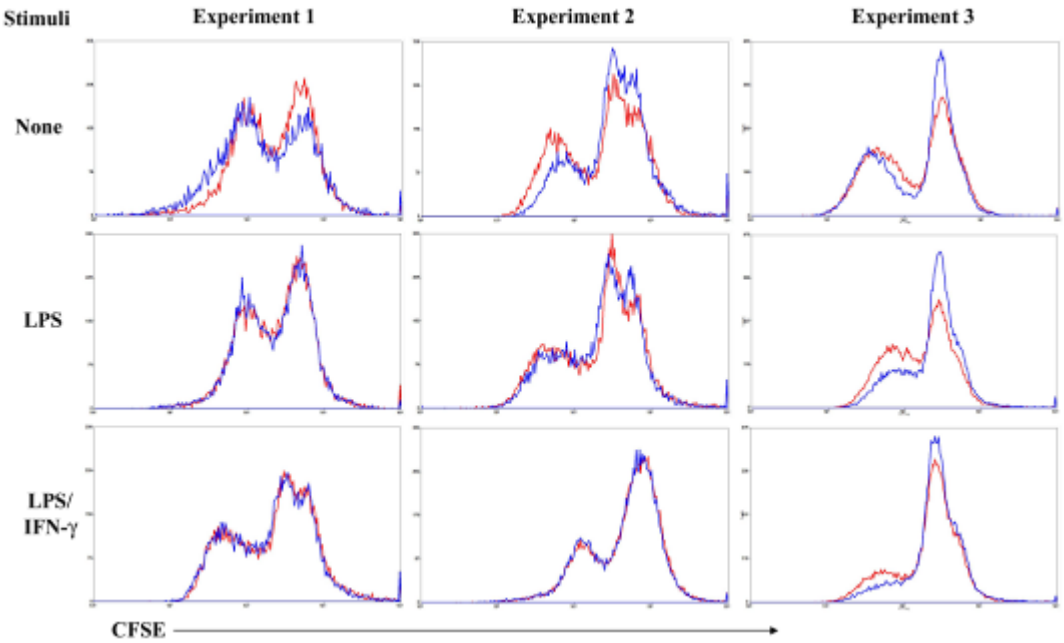

Figure 6.10. Allogeneic naïve CD4 T cell proliferation in response to normal DCs and C1qDCs. Naïve CD4 cells were isolated from PBMCs by negative MACS selection and labeled with CFSE. The cells were then co-cultured with normal DCs (blue) or C1qDCs (red) for 7 days with LPS or LPS/IFN-γ stimulation or without stimulation (none). The T cells were gated, based on forward and side scattering, in flow cytometric analysis for the level of CFSE. Three independent experiments were presented which show that the two DC types induce similar T cell proliferation.

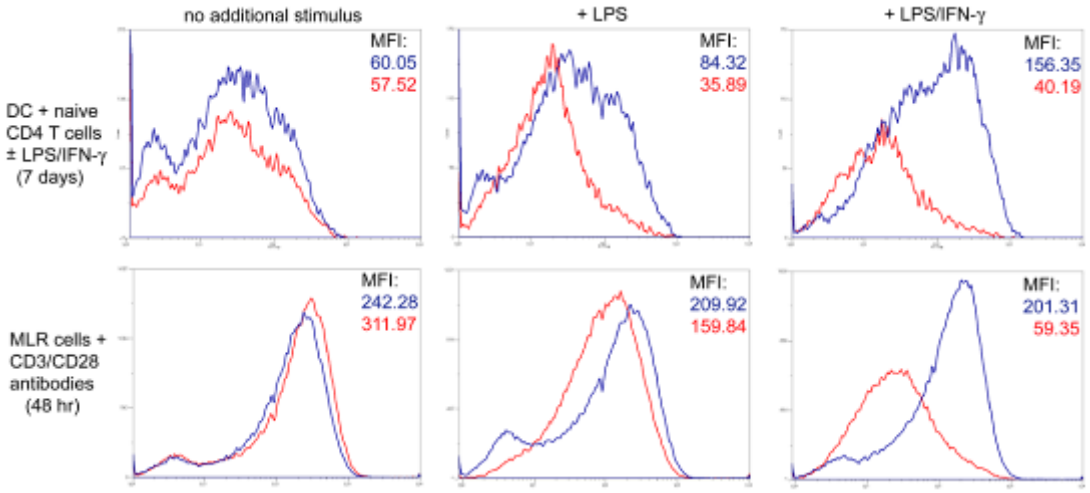

Figure 6.11. CD25 induction by C1qDCs and normal DCs on naïve CD4 T cells. Upper panels: In 96-well plates, $4x10^4$ DCs were co-cultured with $2x10^5$ naïve CD4 T cells (0.2 ml/well), in the presence or absence of LPS or LPS/IFN-γ. At day 7, cells were stained for CD4 (Pacific blue) and CD25 (PE) and CD4 cells were gated for CD25 detection. Lower panels: At day 7, cells were washed and re-stimulated for 2 days with CD3 and CD28 antibodies in 96-well plates (0.2 ml/well). Cells were similarly analyzed for CD25 expression on CD4 cells. Red histograms, CD4 cells primed by C1qDCs in primary MLR; blue histograms, CD4 cells primed by normal DCs. The results shown are representative of 3 independent experiments.

6.9 C1qDCs exhibit greater ERK, p38 and p70 S6 kinase activation than normal DCs

How C1q induced these C1qDC properties is unclear. Without knowledge of the C1q receptors involved, we examined the signaling properties of C1qDCs that might impact their IL-10, IL-12 and IL-23 expression. ERK and PI3K signaling can regulate these cytokines and generally enhance IL-10 but reduce IL-12 and Il-23 production (Yi *et al.*, 2002; Fukao and Koyasu, 2003; Agrawal *et al.*, 2006; Yang *et al.*, 2006). The p70 S6 kinase (p70S6K) has been implicated in the cytokine regulation by PI3K (Fukao and Koyasu, 2003; Yang *et al.*, 2006). C1q may indeed

activate ERK and PI3K through the CD91 and gC1qR receptors (Waggoner *et al.*, 2005; Jehle *et al.*, 2006).

DCs were stimulated for 10, 30, 60 and 120 min with LPS. ERK phosphorylation was examined by Western blotting. Significantly greater ERK activation was detected in C1qDCs as compared with normal DCs (Fig. 6.12). We found that another MAP kinase p38 also showed stronger activation in C1qDCs which supports a recent report that, like ERK, p38 signaling also enhances IL-10 but inhibits IL-12 production (Jarnicki *et al.*, 2008). JNK activation was not significantly different between the two DC types. PI3K signaling was examined by detecting p70S6K and Akt kinase activation and C1qDCs showed higher p70S6K phosphorylation than normal DCs. However, Akt activation appeared slightly lower in C1qDCs and how this might be related to IL-10, IL-12 and IL-23 production by C1qDCs is unclear. However, a stronger p70S6K activity in C1qDCs is consistent with the high IL-10 but low IL-12 and IL-23 production by these cells (Fukao and Koyasu, 2003).

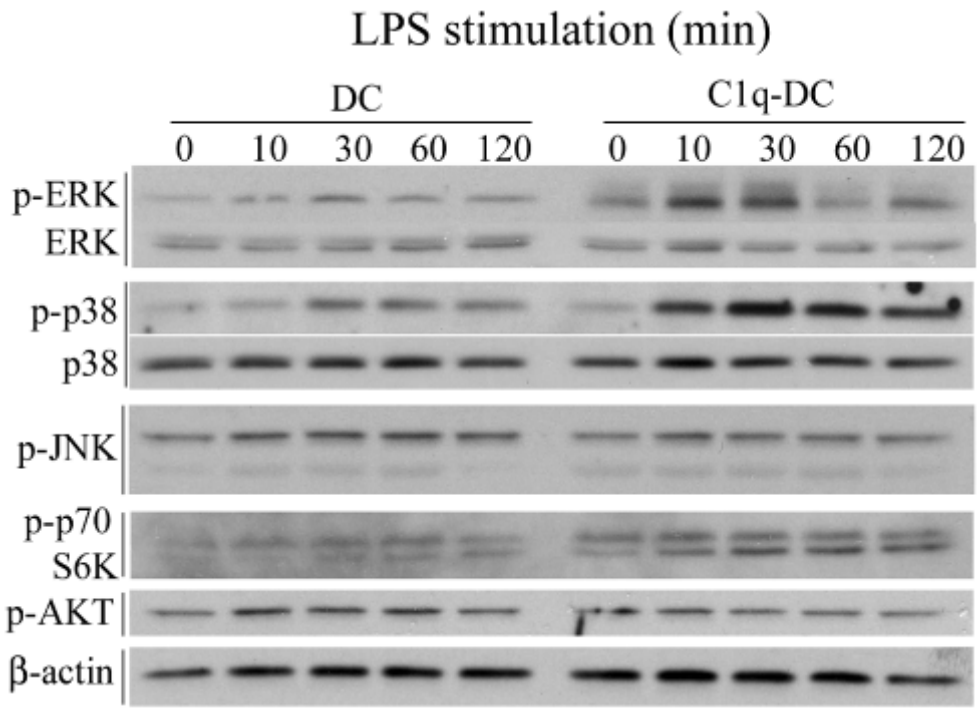

Figure 6.12. C1qDCs exhibited stronger ERK, p38 and p70S6K activation than normal DCs. Normal DCs and C1qDCs were resuspended at $1x10^6$/ml and then seeded in 24-well plate ($0.5x10^6$/well). The cells were stimulated with LPS for 10, 30, 60 and 120 min. At the indicated time-points, the cells were chilled, lysed and subjected to Western blotting to detect phosphorylated (p-) ERK (Thr^{202}/Tyr^{204}), p38 (Thr^{180}/Tyr^{182}), JNK (Thr^{183}/Tyr^{185}), p70S6K (Thr^{389}) and AKT (Thr^{308}). As controls, total ERK, p38 and β-actin were also detected on the blots.

6.10 Inhibition of ERK renders C1qDCs similar to normal DCs in its IL-10 and IL-12 production

The relevance of elevated ERK activation in C1qDCs to its IL-10 and IL-12 profile was assessed by ERK inhibition in these cells. C1qDCs and normal DCs were pre-treated with the MEK1 inhibitor PD98059 before further stimulation. As shown in Fig. 6.13A, this reduced IL-10 production by C1qDCs but not normal DCs. As a result, the two DC types produced similar levels of IL-10. On the other hand, PD98059 treatment increased IL-12p70 production by both DC types but it caused a

greater increase in C1qDCs. As a result, C1qDCs were drawn nearer to normal DCs in IL-12p70 production; from a 7-fold difference to < 2 fold (Fig. 6.13B). PD98059 similarly restored C1qDC production of IL-12p40 close to the level of normal DCs (Figure 6.13C).

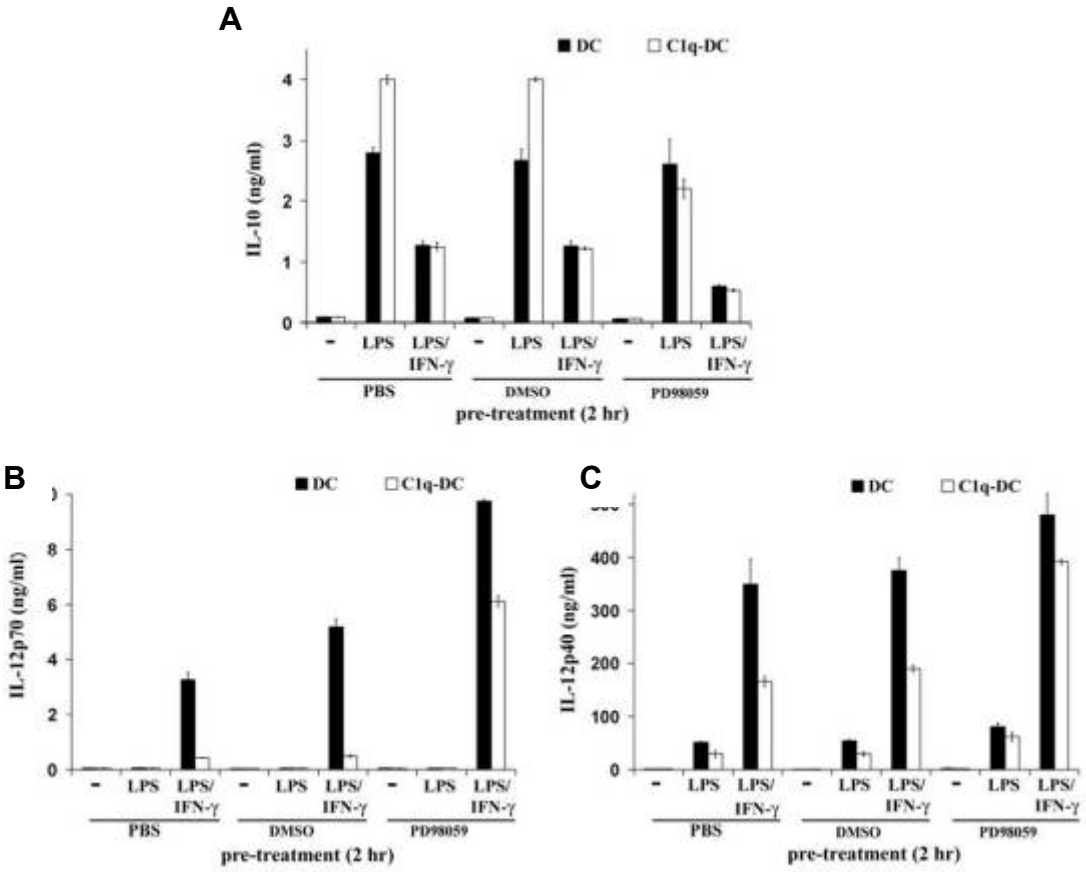

Figure 6.13. ERK inhibition partially restored the inferior IL-12 production in C1qDCs and abrogated its superior IL-10 production. Normal DCs and C1qDCs were pre-treated for 2 hr with the MEK1 ERK kinase inhibitor PD98059 (40 μM), the vehicle DMSO or PBS before stimulation with LPS or LPS/IFN-γ for 24 hr. As a control, the cells were not stimulated (-). **(A)** IL-10, **(B)** IL-12p70 and **(C)** IL-12p40 production was determined by ELISA. The experiments were carried out in triplicates and data was presented as means ± SD.

Chapter 7 Discussions

7.1 Assays for analyzing C1q production in human monocyte derived DCs

We set out to investigate the regulation of C1q production and to find novel factors that may influence C1q production, especially in the context of SLE. We have established a system to screen a panel of ligands and drugs for their ability to upregulate or downregulate C1q production. In this system, we exposed human monocyte-derived DCs to these stimulants and analyzed their ability to influence C1q production. Our analysis of C1q production began at the mRNA level by using real-time PCR, then we studied the cellular protein levels by using Western blot and finally we quantitated secreted C1q by ELISA.

Our culture system consistently yielded highly pure monocytes, which did not express C1q. The monocytes were differentiatiated into functional DCs, which then expressed C1q mRNA and secreted C1q into the culture supernatant. We analyzed the ability of the different stimuli to influence C1q production in DCs over 2 time-points (2 and 2 + 2 days). C1q mRNA and protein levels were mainly not affected over a short stimulation period of less than 1 day. The effects of stimulations on C1q production became clearer only after 2 days of stimulation and were even more apparent after 4 days (Figs. 4.3, 5.3 and 5.4). The increase in C1q protein correlates well to the mRNA levels for unstimulated and IFN-γ stimulated DCs.

Washing of the cells after the first 2 days of stimulation and culturing for a further 2 days mimics the *in vivo milieu,* where DCs are exposed to inflammatory stimuli at the site of stimulation, mature and migrate away from their niches to lymphoid tissues (Randolph *et al.*, 2005). DC maturation results in a profound change, where it upregulates co-stimulatory molecules like CD40, CD80 and CD86, increases its antigen presentation capability by upregulation of MHC I and MHC II and express surface CCR7 (Sanchez-Sanchez *et al.*, 2006). The chemokine receptor CCR7 guides the migratory DCs to the lymph nodes via the lymphatic system.

Many of the previous studies on C1q production based their conclusions solely on mRNA expression or total cellular protein levels that may not represent the secreted bioactive fraction of C1q. This fraction can be quantitated by the ELISA assay and this assay was crucial in detecting the suppressed C1q secretion following prolonged IFN-α stimulation (Chapter 5). Another possible disadvantage in the mRNA and Western blot studies is that these analyze only the individual chains of C1q. Western blotting is usually performed on proteins resolved under reducing SDS-PAGE and thus the C1q macromolecule is resolved into its 3 constituent A-, B- and C-chains. Therefore, these two methods would still give results even if individual C1q chains were expressed but not assembled into the complete C1q molecule.

Our analysis of C1q production using ELISA, intracellular flow cytometry and confocal microscopy were carried out using a monoclonal anti-C1q globular domain antibody. We observed that this antibody did not detect C1q in Western blots of proteins separated under reducing conditions (data not shown). We strongly suspect

that this antibody detects only the full C1q protein, and thus the assays using this antibody should detect only the fully assembled C1q molecule and not the individual A-, B- and C-chains. The intracellular flow cytometry detection of C1q is a novel method and has not been used before to our knowledge, although there are reports of antibodies able to detect membrane-bound C1q (Kaul and Loos, 2001). However we could not detect C1q on cells without permeabilization (data not shown). Our novel intracellular flow cytometry detection of C1q may possibly be applied to future studies in studying C1q synthesis and assembly.

As we used primary monocytes obtained from healthy donors of a diverse background in the generation of DCs, the C1q levels in cell culture supernatant as detected by ELISA can fluctuate widely between ~10 ng/ml to ~250 ng/ml after 2 days of culture without any stimulation. Nevertheless, the properties of various stimulants in upregulating or downregulating C1q production remain identical across DCs from various donors.

7.2 Regulation of DC production of C1q by microbial and autoimmune disease factors

With the ELISA system, we tested the influence that many microbial stimuli, steroid drugs, hormones and cytokine/chemokines may have on affecting C1q production in DCs. By focusing on a single cell type for analysis, in this case DC, we should eliminate conflicting results, such as when the same stimulant used on different cell

types giving different results. The list of stimulants used was curated loosely based on a previous review of how C1q production is regulated (Lu *et al.*, 2008).

Stimulation of TLR3, TLR5, TLR7/TLR8 and NOD1/NOD2 had minimal effects on C1q levels. Stimulation of TLR2 with its various ligands suggests that this receptor could potentially upregulate C1q production, the degree of which depends on the ligands used. FSL-1, Pam2CSK4 and Pam3CSK4 all stimulate TLR2 in synergy with either TLR6 or TLR1 and these 3 ligands upregulated C1q production more strongly than ligands stimulating TLR2 alone (LTA and PGN). Zymosan and CpG DNA are TLR ligands that suppressed C1q production.

There are notable differences in the regulation of C1q expression based on our results and what other groups have reported. For example, one recent study using mouse BMDC has found that LPS maturation abrogates C1q production at both the mRNA and secretion levels (Castellano *et al.*, 2010). An earlier study by the same group had similar results when moDC was used. TNF-α, LPS and CD40L were used to stimulate the DCs, and it was found that secreted C1q levels were reduced by about one-third for both TNF-α and LPS and by two-thirds for CD40L (Castellano, 2004). Strikingly, they observed that mature DCs similar to our 2 + 2 days culture completely stopped producing C1q. TNF-α also reduced C1q production in our study. But in contrast, our study showed that CD40L had no significant effect while LPS either had no effect or it could increase C1q secretion by about two-fold (Fig. 3.6). Nevertheless we never observed any downregulation of C1q with LPS stimulation for both 2 days and 2 + 2 days culture across many independent experiments. Another study using moDCs found that LPS increased C1q secretion

(Baruah *et al.*, 2006). Upon closer inspection of the system employed by Castellano *et al*, it was noted that their stimulations were performed together with the DC differentiation cytokines IL-4 and GM-CSF, whereas in our experiment and that of Baruah *et al*, the DCs were stimulated with just LPS and no additional cytokines. This discrepancy suggests that the macro-environment of the DCs could influence the expression of C1q regulated by a same agent. The same study by Baruah *et al* found that PGN and LTA increased C1q secretion by about 2 times after 2 days, but we observed minimal difference in C1q levels after 2 days, although over 2 + 2 days the C1q levels were increased. Possibly, the effects of these two ligands are only apparent after the DCs are fully matured.

Both osteoprotegerin (OPG) and thymic stromal lymphopoietin (TSLP) downregulated C1q production in DCs. OPG is a TNF receptor family member. It is a decoy receptor for the osteoclastogenic RANK ligand and thus it inhibits osteoclast formation. No studies have linked OPG to SLE pathogenesis, although it was observed that serum OPG levels of SLE patients were higher than healthy controls (Kwok *et al.*, 2009). TSLP is a cytokine mainly expressed by barrier surface epithelial cells and is a potent activator of DCs, with a resulting Th2 polarizing phenotype that can promote allergy (Ziegler and Liu, 2006). So far, there are no studies linking TSLP to SLE. IFN-γ clearly upregulated C1q secretion, whereas IFN-α suppressed C1q production only after 2 + 2 days. Most of the other cytokines had minimal effect on C1q production, except notably IL-10 increased C1q production after 2 + 2 days. As to whether this could be attributed to the anti-inflammatory nature of IL-10 remains to be studied.

Of the steroid drugs tested, none of them dramatically affected C1q secretion, which differed from observations that they promoted C1q production in macrophages (Trinder *et al.*, 1995; Armbrust *et al.*, 1997; Walker, 1998). This could be a cell-type or species specific issue, as the 3 studies respectively used rat liver macrophages, mouse peritoneal macrophages and THP-1 differentiated macrophages as compared to our study using human moDCs. We included female hormones into this study as about 90% of SLE patients are females and pregnancy and menstrual cycle can influence the disease activity in SLE patients (Kawasaki *et al.*, 2009). Estradiol slightly reduced C1q secretion in 2 days but progesterone showed the same effect for both 2 days and 2 + 2 days cultures. Nevertheless our results here are too preliminary to conclude if female hormones in general could reduce C1q levels in females.

Overall, most stimuli had minimal effect on C1q production. For stimulants that clearly affected C1q production, we chose 3 for further investigation – zymosan, IFN-α and IFN-γ and the results would be discussed in Chapters 7.4 and 7.5.

7.3 Production of C1q by primary DCs

In vivo, human DCs have been reported to produce C1q and C1q was also deposited around the DCs in various tissue sections as reviewed in Chapter 1.4.4. The localized C1q may have regulatory effects on the DCs and this was explored in Chapter 6. The monocyte-derived DCs used throughout this study are recognized to be generated only under inflammatory conditions and not in the steady state (Shortman and Naik, 2007). However they are functionally relevant in the immune

system. For example, inflammatory DCs generated from monocytes during *Listeria monocytogenes* are crucial for the clearance of the bacteria via TNF and iNOS-induced ROS production (Serbina and Pamer, 2006), In colitic mice, monocyte-derived inflammatory DCs that express E-cadherin, the receptor for CD103, promote intestinal inflammation and are detrimental to the host (Siddiqui *et al.*, 2010). The GM-CSF generated mouse BMDC are also inflammatory DCs similar to human moDC (Shortman and Naik, 2007). These two DCs respectively produced the highest levels of C1q among DCs from both mouse and human (Figs. 3.11 and 3.13).

The division of steady state DCs into pDCs and conventional DCs is accepted for both human and mouse. For the mouse, two functionally distinct populations of cDC have been recognized, one with high surface expression of CD8α and the other lacking this marker. $CD8^+$ DCs are $CD11c^+$, $CD11b^{low}$, $Sirp\alpha^{low}$ and functionally these DCs are the major producers of IL-12, are able to capture dead cells and to cross-present different forms of exogenous antigens on their MHC class I molecules and can prime cytotoxic T cells (Villadangos and Schnorrer, 2007). mRNA analysis showed that $CD8^+$ DCs expressed the lowest levels of C1q. Recently, the rare and hard to isolate $CD141^+$ (BDCA-3) human cDC subset was found to closely resemble mouse $CD8^+$ DCs (Villadangos and Shortman, 2010). It expresses the $CD8^+$ DC markers XCR1 and Clec9A, is capable of phagocytosing dead cells and cross-presenting cell-associated and soluble antigens and produce IL-12. Whether the human $CD141^+$ DCs express low levels of C1q in parallel to mouse $CD8^+$ DCs was not studied.

The mouse $CD8^-$ DCs show high expression of MHC class II antigen processing and presentation genes, and are generally known to induce $CD4^+$ T helper cell response and promote humoral immunity in response to bacteria or extracellular parasite infection. Besides being $CD8\alpha^-$ and $CD11c^+$, they are also $CD11b^+$ and $Sirp\alpha^+$ and expressed the highest levels of C1q mRNA of all splenic DCs, but lower than C1q levels in BMDCs. Comparative genomics studies coupled with functional similarities have suggested that human myeloid $CD1c^+$ (BDCA-1) DC is equivalent to mouse the $CD8^-$ DC (Crozat *et al.*, 2010). $CD1c^+$ DC produced C1q but at lower levels than moDCs. Both human $CD1c^+$ DC and mouse $CD8^-$ DC produced higher levels of C1q than pDCs. Although we detected low levels of C1q mRNA in human pDCs, we failed to detect secreted C1q in pDC culture supernatants. Secreted mouse C1q was not measured in this study.

Our results that showed the expression of C1q in different DC subtypes in both mouse and human is novel. The relative C1q production level seems to be unique to each DC subtype and could serve as a novel marker for differentiating DC subtypes in both mouse and human. Further analysis of C1q expression in other types of DCs such as the migratory Langerhans cell and dermal DCs and the $CD11b^+CD103^+$ DCs of the gut remains to be conducted.

7.4 Dectin-1 engagement is a novel mechanism that holistically downregulates C1q production – implications in SLE pathogenesis resulting from fungal infections

Dectin-1 (Clec7a) is a member of the C-type lectin receptor (CLR) family, and is expressed mainly on innate cells such as macrophages, neutrophils, and DCs. Dectin-1 is involved in the recognition of a broad range of fungal pathogens, including *Candida albicans*, *Aspergillus fumigatus* and *Pneumocystis carinii*, and it promotes protective antifungal immunity against *C. albicans* that requires Th1 and Th17 responses (Taylor *et al.*, 2007; Gringhuis *et al.*, 2009). Structurally, Dectin-1 is a glycosylated type II transmembrane receptor with a single extracellular C-type lectin domain connected by a stalk to a transmembrane region and a cytoplasmic tail (Kerrigan and Brown, 2010). Dectin-1 binds β-1,3-linked glucose oligomers, and this stimulation can induce many cellular responses including the respiratory burst, DC maturation, ligand uptake through phagocytosis and endocytosis, the production of arachidonic metabolites and various cytokines and chemokines such as TNF, CXCL2, IL-23, IL-6, IL-2, and IL-10 (Reid *et al.*, 2009).

Zymosan is a yeast cell wall derivative commonly used as a ligand to represent fungal pathogens and can activate both TLR2 and Dectin-1 through its β(1,3)-glucans. With respect to C1q production, zymosan appears to act through Dectin-1 to reduce C1q expression by DCs whereas most of the TLR ligands showed no such inhibitory effects. For example, with LPS, C1q production was enhanced and DCs stimulated with all other TLR2 ligands eventually showed enhanced C1q production (Chapter 3.4). Curdlan, which contains Dectin-1–stimulatory β(1,3)-glucans but lacks TLR-stimulatory motifs (Gringhuis *et al.*, 2009) was also found to mediate

suppression of C1q production. Either mode of Dectin-1 stimulation downregulated C1q mRNA and intracellular protein production and secretion, and this was not due to increased cell death.

Beyond the TLR family, Dectin-1 was the first PRR found to induce its own intracellular signaling (Reid *et al.*, 2009). β-glucans engagement of Dectin-1 leads to Src family kinases phosphorylating the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM)-like motif and thus providing a docking site for Syk. The components of the signaling pathway have yet to be fully elucidated. Downstream of Syk, caspase recruitment domain 9 (CARD9) is known to assemble with BCL10 and MALT1 to form a complex linking to the canonical NF-κB pathway. There are reports of a Syk-dependent, but CARD9-independent pathways of Dectin-1 activation, and this leads to the induction of ERK and the production of cytokines such as IL-10 and IL-2 (Dillon *et al.*, 2006; Slack *et al.*, 2007). Our efforts to identify the specific Dectin-1 signaling pathway or effect that mediates its inhibition on C1q production were unsuccessful. This includes blocking Syk signaling, inhibition of arachidonic acid release and ROS production.

Dectin-1 mediated phagocytosis in macrophages acts in a Syk-independent pathway and although this response still requires the ITAM-like motif of the receptor, it is still largely uncharacterised (Brown, 2006). Recently, Dectin-1 was recognized as the first PRR known to engage the non-canonical NF-κB pathway. This requires a Syk-independent pathway involving the serine–threonine kinase Raf-1, which integrates with the Syk pathway at the level of NF-κB (Gringhuis *et al.*, 2009). Another recent study found that NFAT transcription factors were activated by

Dectin-1 and this requires intracellular Ca^{2+} flux coupled to the phospholipase Cγ pathway (Xu *et al.*, 2009b). Both pathways mediate Dectin-1 induced cytokine production. Blocking of both pathways through the use of a Raf-1 inhibitor and through intracellular Ca^{2+} chelation did not abrogate the inhibitory effects of Dectin-1 on C1q production. Calcineurin activation downstream of Ca^{2+} influx was recently shown to mediate the NFAT activation (Greenblatt *et al.*, 2010), but this pathway could be excluded from our system as it involves Ca^{2+} influx.

In a *C. albicans* mortality study of various knockout mice including $C1q^{-/-}$, complement factor B and C2 ($Bf/C2^{-/-}$) and MBL-A and MBL-C ($MBL\text{-}A/C^{-/-}$), C1q-/- mice had the lowest mortality rate of 15% compared to >90% and 40% for the other two knockout mice respectively, with wild-type mice showing just 4% mortality (Held *et al.*, 2008). This suggests for a stronger contribution from both MBL and alternative pathways (via factor B) than C1q-mediated classical pathway for controlling *Candida* infection. In addition, it was shown that MBL and C3, but not C1q, act as recognition molecules for *C. albicans* in the vagina and there is an association between reduced levels of vaginal MBL and recurrent vulvovaginal candidiasis (Pellis *et al.*, 2005). With less importance of C1q compared to other innate immune components in clearing fungal infection, suppression of C1q production by fungal stimulation of Dectin-1 does not appear to be an immune evasion mechanism employed by fungal pathogens.

C. albicans is a common constituent of the normal flora of the oropharyx and the gastrointestinal tract of healthy individuals and of the female lower genital tract. However, under predisposing conditions this colonization may lead to

mucocutaneous and also invasive infection. A clue to a possible function of Dectin-1 in SLE is that its patients are highly susceptible to infections from common microorganisms and also to opportunistic infections. Infection accounts for 20–55% of all deaths in SLE (Khalifa *et al.*, 2007). *C. albicans* was the most frequent opportunistic organism identified in fatal infection in adult SLE (Hellmann *et al.*, 1987). This is due to immunological aberrations in SLE patients, both from the disease itself and from the regiment of immunosuppressive treatment of the disease. Some possible aberrations include dysfunctional phagocytic cells, defects in cellular immunity, reduced IgG production, low complement levels and ineffective elimination of microorganisms by reticuloendothelial systems and spleen (Kim *et al.*, 2009).

With high infectious fungal infection rates in SLE patients, could the inverse be true? Fungal infection activates Dectin-1 and this could result in suppression of C1q production by DCs and macrophages, potentially leading to SLE pathogenesis. It is well-known that in active lupus, serum C1q level is reduced. Clinically, individuals who are genetically susceptible to SLE or have developed SLE could encounter accelerated disease or suffer from SLE flares due to infections by fungi and other pathogens that can activate Dectin-1 such as mycobacteria. Another remote possibility is females, who constitute about 90% of SLE patients, could have the higher exposure to *Candida* via their lower genital tract acting as an environmental factor leading to their higher disease predisposition. The implicated connection between reduced C1q expression and fungal infections can help in the understanding of environmental factors leading to SLE development.

7.5 IFN-α, an important SLE pathogenic factor, downregulates C1q secretion

IFN-α is primarily produced by pDCs in response to infection or IC stimulation whereas IFN-γ is produced predominantly by T cells, natural killer (NK) cells and NKT cells following inflammatory activation. Generally IFN-α is a poorer inducer of DC maturation as compared to TLR stimuli. IFN-α activated DCs shows a high expression of the maturation marker CCR7 (Dauer *et al.*, 2006), and can upregulated the expression of HLA-DR, CD80, CD86, but not CD83, (Luft *et al.*, 2002). Functionally, IFN-α can lead to the induction of CTL responses, $CD8^+$ survival and enhanced autoantibody production via DC induction of class-switching (Honda *et al.*, 2005).

The roles of C1q as a suppressor- and IFN-α as an enhancer- of SLE pathogenesis were previously discussed in Chapters 1.3 and 1.4. A link between C1q and IFN-α has not been firmly established yet, and the two could interplay in two ways. Firstly, C1q can possibly suppress IFN-α production and thus limiting its pathogenesis in SLE. Secondly and in reverse, IFN-α could negate the suppressive effects of C1q by inhibiting its production and thus tilting the balance towards SLE progression. In agreement with the first hypothesis, it was recently reported that C1q reduced CpG and IC induced IFN-α production by pDC (Lood *et al.*, 2009). In our study, we test the second hypothesis that type I IFN may have strong regulatory effects on C1q production by DC, and also to compare the effects of type I IFN with the type II IFN.

Using DCs, we showed that while IFN-γ enhances C1q levels in culture supernatant, IFN-α attenuates C1q secretion. The treatment of DCs with IFN-γ increased C1q production at the transcriptional, protein synthesis and secretion levels as assessed by mRNA, Western blotting and ELISA assays in both 2 days and 2 + 2 days stimulations. With respect to IFN-α, we observed a strong suppression in secreted protein levels in culture supernatant after 2 + 2 days of stimulation, although not much effect was observed within the first 2 days of stimulation. The downregulation of C1q production does not appear at the transcriptional and translational levels as C1q mRNA and cellular C1q were both increased upon IFN-α stimulation for both 2 days and 2 + 2 days. This is in contrast to the decrease in secreted C1q as measured by ELISA upon IFN-α stimulation and thus the suppression of C1q production occurs at the secretion level. We showed that increased cell death with IFN-α stimulation was not the reason behind this.

We then used confocal microscopy to localize the intracellular compartments of C1q. IFN-α stimulation upregulated C1q expression, but the C1q was found to be mainly trapped in the ER instead of being trafficked to the Golgi for secretion. Clearly, IFN-γ stimulated DCs have less C1q localized in the ER and more in the Golgi apparatus, indicating that they were ready to be packaged and secreted. The mechanisms behind this disparity between the two IFN treatments are unidentified in this study. By understanding the network of protein secretion from ER to Golgi, it is possible to identify the proteins that package C1q into vesicles for transport to the ER-Golgi intermediate compartment of the *cis*-Golgi network that could be affected after IFN-α exposure (van Vliet *et al.*, 2003). There are also regulatory mechanisms in the ER that ensure proteins are properly post-translationally modified and folded

before transport form ER to the Golgi network. Although we believe that the monoclonal antibody against the C1q globular domain detects the fully assembled C1q protein in the ER, it is still possible that the collagenous domain may not be properly assembled and its transport to the Golgi could be halted. Hydroxylation of proline and lysine resides are important for stabilization of the collagen triple helix, and also for C1q production (Muller *et al.*, 1978; Mocharla *et al.*, 1987). Hence, another possibility is IFN-α effecting the expression or functionality of chaperones and hydroxylases in the ER that are required for C1q assembly.

When the two IFNs operate alone, they have opposite effects on modulating C1q production from DCs. However, when the two stimuli were added concurrently to DCs, the C1q-enhancing signals from IFN-γ dominated over the C1q secretion-suppressing signals from IFN-α. There are two potential mechanisms behind this. IFN-α and IFN-γ together could synergize to upregulate C1q expression and protein translation, such that even though its secretion is generally affected, there are still more C1q secreted than with IFN-α treatment alone. The other potential mechanism is that IFN-γ can abrogate the dysregulation of ER functions that trapped C1q in the ER following IFN-α stimulation.

7.6 C1q conditions the differentiation of DCs with immunosuppressive properties, possibly raising the threshold of immune activation required for autoimmunity

The initiation of the complement classical pathway requires recognition of a microbial or self antigen by IgG or IgM antibodies. This is followed by C1q binding, and the binding is believed to cause a conformational change to C1q that triggers activation of the C1 catalytic subunit, the Ca^{2+}-dependent tetramer and ultimately activating the entire complement cascade (Kishore *et al.*, 2002). As reviewed in Section 1.4.4, C1q deposits in the ECM around DCs and it is possible that the C1q have direct effects on DC differentiation and functionality. The C1q deposited in normal ECM in the absence of autoantibodies is not expected to activate complement and inflammation, as the mechanisms that attach it onto ECM may not cause the conformational change similar to activation on IgG binding. Instead, excess soluble C1q during DC culturing rendered DCs refractory to maturation stimuli (Castellano *et al.*, 2007). We thus sought to examine how surface-deposited C1q may regulate DC development from monocytes.

The presence of immobilized C1q neither interfered with the monocyte differentiation to DCs nor did it influence the survival of DCs. We obtained roughly equal number of normal DCs and C1qDCs after 6 days of monocyte culture. Both types of DCs were similar in surface MHC and co-stimulatory molecules (CD40, CD80 and CD86) expression. On stimulation, the mature forms of both DCs upregulated the co-stimulatory molecules CD40, CD80 and CD86, MHC I and II and the maturation markers CD83 and CCR7 to similar levels. Thus, the differentiated C1qDC were phenotypically and functionally immature like normal

DCs. As deposited C1q did not significantly modulate the expression of the essential molecules for T cell stimulation, C1qDCs were also similar to normal DCs in stimulation naïve T cell proliferation.

C1qDCs showed reduced adhesion to cell culture wells over the differentiation process. However, after the DCs were harvested to be reseeded for stimulation, we observed that C1qDCs could adhere and spread on the new cell culture surfaces and are now morphologically similar to normal DCs. We have stained normal DCs and C1qDCs for integrins and some surface receptors known to mediate cell adhesion, including integrin αV, β1, β2, α2β1, α5β1, αVβ3, αVβ5, E-cadherin and complement receptor 3, and did not find notable differences in the expression between both the DCs (data not shown). Thus the mechanism behind reduced adhesion of C1qDCs over the differentiation and its functional significance remains unknown.

Nonetheless, when further analyzed, C1qDCs show major functional differences compared to normal DCs. C1qDCs are better at phagocytosis of ACs (Fig. 6.3), produce more IL-10 but less IL-12, IL-6 and IL-23 (Fig. 6.4) and consequently are weaker in polarizing CD4 T helper cells to a Th1 and Th17 phenotype (Figs 6.6 and 6.7). The production of less TNF-α by C1qDCs also suggest it is less inflammatory in general when stimulated. C1qDCs and normal DCs also show disparate T cell activation properties in response to maturation stimuli. In the presence of maturation stimuli, normal DCs showed increased ability to activate naïve T cells (as adjudged by CD25 upregulation) while under the same conditions, C1qDCs showed reduced CD25 induction on these cells (Fig. 6.11). It suggests that both DCs are equal in T

cell stimulation in the steady state when self-antigens dominate in tissues, and during infection and inflammation, DCs conditioned without C1q are better at activating T cells. This would help in better clearance of infection, but unabated activation of the T cells could lead to breach of tolerance.

We then attempted to understand the mechanisms leading to the characteristic higher IL-10 but lower IL-12 and IL-23 production by C1qDCs, as compared with normal DCs. How C1q induced these DC properties seems to involve the conditioning of these cells for elevated MAP kinase ERK, p38 and phosphoinositide 3-kinase (PI3K) signaling in the regulation of IL-10, IL-12 and IL-23. It was shown that ERK activation enhances IL-10 but inhibits IL-12 production (Yi *et al.*, 2002; Agrawal *et al.*, 2006). Likewise, p38 activation also enhanced IL-10 while inhibiting IL-12 production (Jarnicki *et al.*, 2008). PI3K activation inhibits both IL-12 and IL-23 production while also enhancing IL-10 production, and the latter involves the activation of the p70 S6 kinase (p70S6K) (Fukao and Koyasu, 2003; Yang *et al.*, 2006). From our results on MAPK phosphorylation, enhanced ERK signaling seems to be the most relevant in instilling C1qDCs with its unique cytokine profile compared to normal DCs, as was directly demonstrated by using the MEK inhibitor PD98059. To a large extent, it restored C1qDC production of the two cytokines to levels comparable to normal DCs. How increased p38 and p70S6K activation in C1qDCs may be interconnected with increased ERK activation is uncertain, but their reported relevance to IL-10, IL-12 and IL-23 production strongly suggests involvement of these signaling pathways in C1qDCs. How C1q tuned DCs for elevated ERK activation remains to be determined.

Kuchroo and colleagues described a 3-step process for Th17 induction: (*1*) TGF-β plus IL-6 induces the differentiation of Th17 cells, (*2*) IL-21 amplifies the frequency of Th17 cells, and (*3*) IL-23 terminally differentiates and stabilizes the phenotype of Th17 cells (Korn *et al.*, 2009). RORγt, and RORα are transcription factors that define the Th17 transcriptional program. Thus, the high IL-6 and IL-23 production by normal DCs is more permissive for Th17 development than C1qDCs, which can be amplified by positive feedback from IL-21 produced by Th17 cells.

In SLE, the protagonist role of type I IFN was strongly established. However, the roles of Th1 and Th17 cytokines in SLE have not been consistently established. In mice, transgenic IFN-γ over-expression causes lupus-like autoimmunity (Seery *et al.*, 1997). With lupus-prone NZB/NZW F1 mice, either breeding to IFNγ$^{-/-}$ background or the use of anti-IFN-γ antibodies reduced disease severity in both cases (Jacob *et al.*, 1987; Balomenos *et al.*, 1998). A study using a lupus-like disease mouse model generated by surface expression of an ER HSP gp96 showed no involvement of IL-23 nor expansion of Th17 population (Dai *et al.*, 2007), although another study using the MRL/*lpr* mice lupus model detected an expansion of the Th17 cell population (Yang *et al.*, 2009). Thus far, there are no studies investigating the mechanisms by which Th17 cells can be pathogenic in SLE mice models.

The data in human studies are less clear, and only clinical studies on the prevalence of Th17 cells/cytokines in SLE patients were conducted until now. One older study detected that IL-17 levels were increased in serum from systemic sclerosis patients, but not in that from SLE patients or healthy donors (Kurasawa *et al.*, 2000).

However, more recent studies have indicated otherwise. Serum IL-17A level and Th17 transcription factor RORγt mRNA in SLE patients were higher than that of controls (Chen *et al.*, 2010). Another study revealed that the percentage of circulating Th17 cells and their ability to produce interleukin-17A (IL-17A) were increased in samples derived from patients with active SLE, and the number of Th17 cells increased during SLE flare but decreased following steroid treatments (Yang *et al.*, 2009). Henriques *et al* observed a significant decrease in the frequency of Th1 and Tc1 (CD8+ T cells producing IFN-γ) cells, lower frequencies of Treg, and increased Th17 and Tc17 (CD8+ T cells producing IL17) cells in patients with active SLE, when compared with those with inactive disease and healthy controls (Henriques *et al.*, 2010). Another recent study concurred that SLE patients had an increased Th17 cells compared with healthy subjects but the frequency of Th1 cells was similar (Shah *et al.*, 2010).

Studies investigating the natural Treg frequencies in the peripheral blood of patients with SLE have generated controversial results (Bonelli *et al.*, 2010). Although most studies have described decreased proportions of $CD4^{+}CD25^{high}$ Treg in patients with SLE as compared with healthy controls and observed an inverse correlation of Treg numbers with clinical disease activity, other have reported no change or even higher proportions of Treg in patients with SLE and a positive correlation with disease activity. If Tregs do contribute to SLE pathogenesis, in patients with higher Treg detected, it is possible that their Tregs are functionally defective during active SLE (Henriques *et al.*, 2010). Our data indicates that C1qDC does not induce more Treg than normal DCs, which could have been a possible mechanism in normal DCs inducing more Th1 and Th17 cells. However, the increased IL-10 production by

C1qDCs might play a potent inhibitory action on both Th1 and Th2 differentiation (Mills, 2004), in addition to its secretion of less IL-6, IL-12 and IL-23.

Altogether, deposited C1q clearly induced a distinct property in DCs which renders these APCs potent phagocytes for AC or self-antigens. Compared against DCs without C1q conditioning, these DCs are just as potent at steady state T cell stimulation, which however recedes upon microbial or inflammatory activation. This is in agreement with the strong association between C1q deficiency and autoimmunity. We believe that these C1qDCs may better represent endogenous monocyte-derived DCs as these cells produce C1q and deposit C1q in surrounding tissues.

7.7 Final conclusions

Genetic loss of C1q is known to be the strongest susceptibility factor in SLE. During disease progression in patients with no genetic C1q deficiency, suppressed C1q levels due to anti-C1q antibodies (Botto and Walport, 2002), consumption from complement activation and possibly reduced C1q production are all positively related with disease progression.

With the immunoregulatory roles of C1q established, and reduced C1q expression by DCs being a possible mechanism in SLE pathogenesis, we have screened for factors that can suppress C1q production in DCs. We studied 2 novel pathways that suppressed C1q production. Dectin-1 stimulation downregulated C1q production in a conventional manner by reducing C1q mRNA expression and its protein synthesis,

thus less C1q was secreted. On the contrary, IFN-α suppressed DC production of C1q at the secretion level.

Differentiation of DCs in the absence of C1q produced DCs with stronger immunostimulatory properties. In fact, DCs differentiated with C1q or "C1qDCs" may represent DCs in normal individuals with normal C1q levels, and the lack of C1q causes DCs to differentiate towards the "normal DC" phenotype as observed in our study.

Overall, we postulate that with C1q insufficiency, DCs can cause excessive activation of Th1 cells which are pathogenic at the early development of SLE, with CD8 CTL causing excessive cell death that is not optimally cleared and releasing autoantigens. Non-C1q generated DCs are less effective in phagocytosis of ACs, and uncleared ACs can turn necrotic and more inflammatory. This ultimately leads to the breach of tolerance. In subsequent disease flares, tissue inflammation and damage would require Th17 cells, which are efficiently induced by DCs not differentiated with C1q, and also autoantibodies from B cells to generate ICs. Th17 are the effector T cells involved in inflammation induction and autoimmune tissue injury (Korn *et al.*, 2009). This is supported by the observation of Henriques *et al* (2010) that Th1 and Tc1 to Th17 and Tc17 ratios were decreased in active SLE. In fact, IFN-γ production during active SLE could be protective by subjugating the ability of IFN-α to inhibit C1q production (Chapter 7.5). Deficiency of C1q could lead to further inflammation that is unchecked by the suppressive properties of C1q.

7.8 Limitations of this study and future work

As our initial screening study on modulation of C1q production was performed using moDCs, we have ignored contributions by macrophages, the other major APC producing C1q physiologically. Hence, performing the screening study using macrophages may help to answer the discrepancies between our results and that of others who have studied C1q expression in macrophages. The moDCs used in this study are only generated upon inflammation, but they remain the most convenient source of human DCs due to the large numbers that can be obtained (Shortman and Naik, 2007). Mouse bone marrow cells can be differentiated into DCs that resemble the population in steady state spleen by using the fms-like tyrosine kinase 3 (Flt3) ligand (Naik *et al.*, 2005), and it is possible to explore the regulation of C1q production in these DCs to more closely simulate the *in vivo* steady state DCs.

We found that CpG DNA also suppressed C1q production. It is generally acknowledged that moDCs do not express TLR9, the CpG DNA receptor and the major cells expressing TLR9 are pDCs and B cells (Bauer *et al.*, 2001; Jarrossay *et al.*, 2001; Wagner, 2004). However, there are also reports that TLR9 was expressed in moDCs (Hoene *et al.*, 2006). Recently, new intracellular nucleic acid sensors were found, and more are speculated to exist (Takeuchi and Akira, 2010). For instance, plasmid DNA-activated a TBK1-dependent signaling that resulted in type I interferon signaling and this occured even in the absence of TLR9 or Z-DNA binding protein 1, a potential intracellular DNA sensor, suggesting that cytoplasmic DNA is redundantly recognized by as-yet unidentified receptors (Ishii *et al.*, 2008). TLR9 deficient pDCs also mediated type-1 interferon production when stimulated with CpG DNA (Yanai *et al.*, 2009). It would be interesting to study if the observed

downregulation of C1q production by CpG DNA is caused by direct TLR9 engagement, or from some other novel receptors and signaling mechanisms, with implications in bacterial immune evasion and also possibly autoimmunity from C1q insufficiency following its downregulation after TLR9 stimulation.

After discovering that Dectin-1 activation is a novel C1q inhibitory signal, our work remains largely incomplete. We could not delineate the signaling mechanisms and intermediates involved in the process. Although Syk, CARD9, Raf-1, PLC-γ, and NFATs are all implicated in signaling downstream of Dectin-1, the contribution of specific transcription factors to Dectin-1–induced transcriptional responses and the upstream signaling intermediates remains incompletely characterized (Greenblatt *et al.*, 2010). As research on Dectin-1 is only starting to intensify, more signaling pathways could be discovered, and these may be the signaling involved in our observation. A possible option is to analyze the lipid raft components of Dectin-1 activated DCs, as the Dectin-1 receptor together with its downstream signaling molecules were recently found to translocate to the lipid rafts upon stimulation of DCs with zymosan or β-glucan, and this was important for the functionality of Dectin-1 (Xu *et al.*, 2009a). It is also possible that our inhibition on Syk was incomplete, as we could not fully inhibit curdlan-stimulated cytokine production. Using the new Syk inhibitor R406 (Braselmann *et al.*, 2006) with higher specificity and inhibitory constant than piceatannol inhibitor may help. However this is currently an experimental drug for the treatment of rheumatoid arthritis and is not publicly available.

The biochemical properties and structure of C1q has been extensively studied thus far, but the cellular processes that assemble, package and secrete the molecule have not been investigated. Delineating how IFN-α impaired C1q secretion is definitely worthwhile undertaking, as it may lead to better understanding of another mechanism that may contribute to SLE pathogenesis. Does this impairment affect the secretion of any other proteins? We showed that it did not affect secretion of fibronectin. But we need to test a wider panel of molecules to determine if only secretion of C1q is affected, or is the effect more general or restricted to a particular class of molecules. At the molecular level, it would also be interesting to identify possible chaperones and hydroxylases that are involved in C1q assembly, as under pathological conditions they may be impaired and thus affecting C1q production.

Our data on C1q-differentiated DCs with a suppressive phenotype are all *in vitro*. Studying DCs and T cells from $C1q^{-/-}$ and normal mice as they progressively acquire SLE and looking for parallels with our normal DC (more immunogenic and perhaps parallel to DCs differentiated without C1q in $C1q^{-/-}$ mice) versus C1qDCs (have higher activation threshold and probably parallel to DCs developed in the presence of C1q in normal mice) could help to vindicate our data. Examining the Th17 cells in both mice could also indicate whether these cells are pathogenic in SLE or otherwise.

Collectively, our data provides a foundation for further research into the connection between C1q and SLE and possibly other autoimmune diseases.

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